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**CHARACTERIZATION OF INACTIVE AND STRESS-INDUCED  
ACTIVE FORMS OF THE TRANSCRIPTION FACTOR HSF1**

*An analysis at the cellular level*

**MILOS VUJANAC, MSc**

A thesis submitted in partial fulfilment of the requirements of the Open  
University for the degree of Doctor of Philosophy  
In Molecular and Cellular Biology

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DIBIT, San Raffaele Scientific Institute  
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## **DECLARATION**

**This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself. All sources of information are acknowledged by means of reference.**



## **ACKNOWLEDGEMENTS**

The experiments presented in this thesis were done in the Laboratory of Cellular Stress in San Raffaele Scientific Institute in Milano, Italy under the supervision of Vincenzo Zimarino. I would like to thank Enzo for an excellent guidance throughout the studies and especially for his patience and encouragement. I am also grateful to the former and present lab members, my friends: Hanne Jorgensen, Julia Kutsikova, Monica Rimoldi, Angelia Fenaroli, Karin Holm and Thomas Farkas. It was a real pleasure sharing the time with them, in the lab and outside. Angus Lamond, my external supervisor was support during the experimental work and writing of the thesis. I wish to thank the many people, in DIBIT and from all around, for their gifts of materials. Finally, I would like to thank to San Raffaele Scientific Foundation for awarding me a fellowship for the first three years of my studies (1996-1999).

## Abstract

In mammalian cells, the heat shock response is mediated by the heat shock transcription factor HSF1 that forms active, DNA-binding homotrimers during temperature stress. The subcellular localization of the inactive form of HSF1 has been of great interest for the potential implications in signaling in this system.

I have detected the inactive form of HSF1 mostly in the nucleus of diverse mammalian cell lines. However, I have found that HSF1 is not confined to the nucleus, but continuously shuttles between the nucleus and the cytoplasm at a minimum rate of 1 molecule  $\text{sec}^{-1}$ . A possible link of shuttling with the functional state of HSF1 is suggested by the observation that the shuttling cycle is discontinued during mild heat stress and resumes promptly during stress relaxation. A similar block of nuclear export is observed for deregulated mutants of HSF1 that trimerize at 37 °C, suggesting that the trimerization step inhibits an export activity.

By mutational analysis I showed that HSF1 contains an unusual bipartite nuclear localization signal. Ongoing experiments are defining sequence requirements for nuclear export, most probably on a pathway distinct from Exportin-1 as judged from the refractoriness of nuclear export of HSF1 to Leptomycin B.

I discuss a possible role of shuttling in compartment specific modifications of HSF1 or associations with co-factors.

## **Abbreviations**

**HSF - heat shock transcription factor**

**Hsp(s) - heat shock protein(s)**

**HSE - heat shock element**

**NLS - nuclear localization signal**

**NES - nuclear export signal**

**NPC - nuclear pore complex**

**NPc - nucleoplasmin core protein**

**GFP - green fluorescent protein**

**PK - pyruvate kinase**

**LMB - LeptomycinB**

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# INTRODUCTION

## Heat shock response

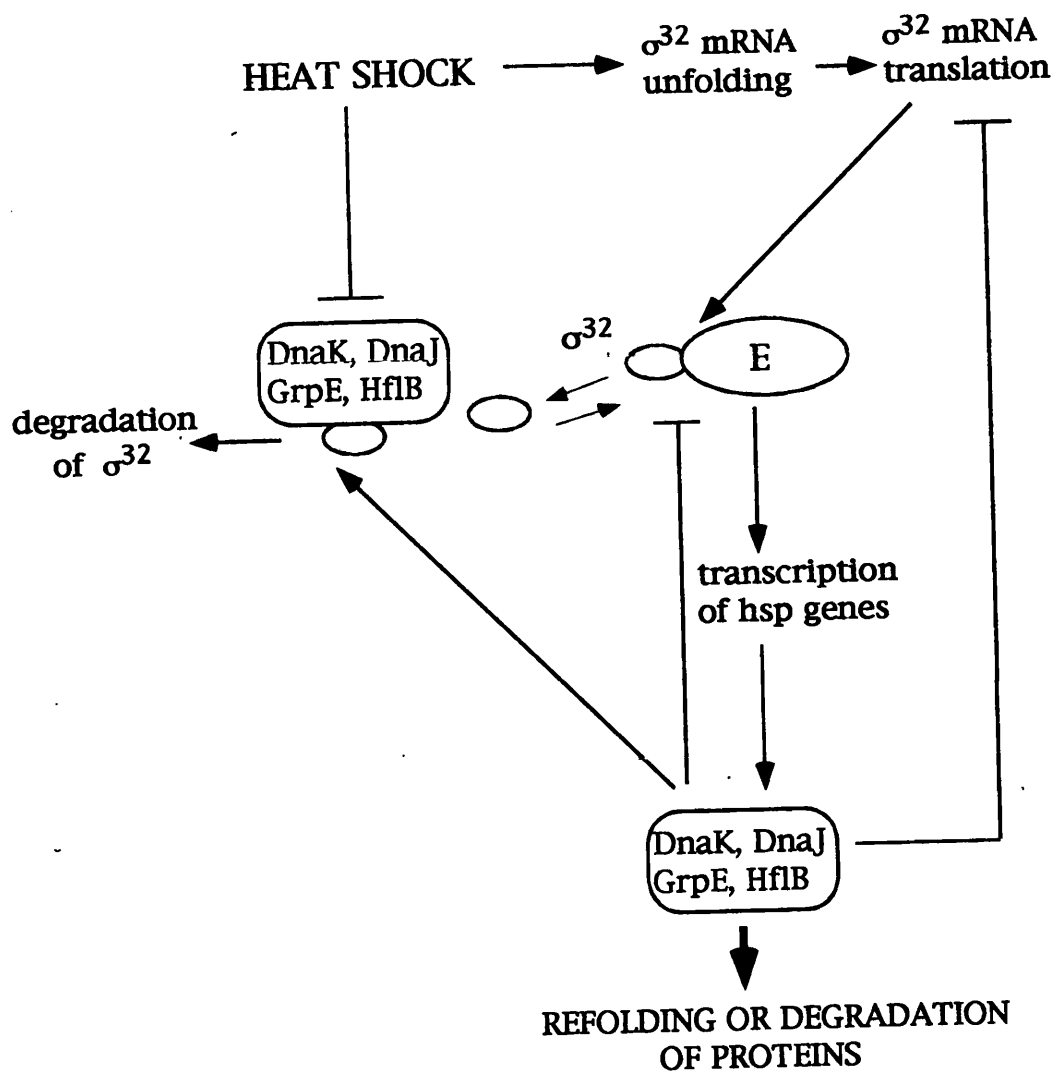
Organisms as diverse as bacteria and man respond to elevated temperature and to a variety of chemical and physiological stresses by expressing a family of highly related proteins referred to as heat shock proteins (hsps). This phenomenon is called the heat shock response and was originally identified in *Drosophila* larvae exposed to elevated temperature or chemicals (Ritossa, 1962). These treatments induced specific puffs on the polytene chromosomes that were found to correspond to heat shock gene loci. Subsequent studies revealed that the expression of hsps in Eukaryotes is inducibly regulated by a variety of conditions that can be broadly categorized in three classes: environmental stress, physiological stress and nonstress conditions (Morimoto *et al.*, 1996). Environmental stress includes exposure of cells to heat shock, heavy metals and chemicals such as metabolic poisons (amino acid analogues and inhibitors of energy metabolism). Physiological stress includes the cellular response induced by bacterial and viral infection, tissue trauma and ischemia or fever and inflammation. The synthesis of hsps can also be modulated under non-stress conditions such as progression through the cell cycle, development and differentiation and by certain oncogenes. It is believed that hsps exert their biological role acting as molecular chaperones

involved in protein folding, transport, higher order assembly or degradation. Therefore, their role during the heat stress is essential to ensure survival by preventing and repairing protein damage caused by denaturation and aggregation (Welch, 1993; Parsell *et al.*, 1994). Moreover, some members of hsp family are synthesized constitutively under normal conditions of growth facilitating correct folding, transport and localization of mature proteins. The fact that hsps are highly conserved in evolution suggests an essential role for cell survival.

## **Regulation of the heat shock response in Bacteria**

In *Escherichia coli*, the genes encoding hsps are under the control of a specific transcription factor,  $\sigma^{32}$ , which is the key regulator of the heat shock response (Georgopoulos *et al.*, 1996).  $\sigma^{32}$  is encoded by the *rpoH* gene and its transcription is not induced when the temperature is raised from 37°C to 42°C. However, at extreme temperatures (>50°C), another sigma factor,  $\sigma^E$ , directs RNA polymerase to one of the four promoters that control expression of *rpoH* (Grossman *et al.*, 1984; Erickson *et al.*, 1987). Under normal growth conditions (37°C),  $\sigma^{32}$  is present at low level, but upon a temperature shift to 42°C,  $\sigma^{32}$  accumulates and directs core RNA polymerase to the promoters of hsp genes (Tilly *et al.*, 1989). Earlier studies have shown that both increased synthesis and stability account for accumulation of  $\sigma^{32}$ . Moreover, the

activity and the association of  $\sigma^{32}$  with RNA polymerase are also subjected to heat shock regulation. Genetic and biochemical evidence revealed that this regulation is achieved through interaction of  $\sigma^{32}$  with DnaK, DnaJ, GrpE and HflB (Straus *et al.*, 1987, 1989). At normal temperature these hsp sequester and target  $\sigma^{32}$  for degradation. During heat stress, non-native proteins would accumulate and titrate DnaK, DnaJ, GrpE and HflB away from  $\sigma^{32}$ . In this way,  $\sigma^{32}$  would be free to associate with core RNA polymerase and target it to the promoters of hsp genes. Since DnaK, DnaJ, GrpE and HflB themselves are under transcriptional control of  $\sigma^{32}$ , there seems to be a negative feedback regulation of the heat shock response in *E. coli* (Figure 1). It has been known that during the heat stress, increased synthesis of  $\sigma^{32}$  is regulated at the level of translation, but only recently, Morita and colleagues (Morita *et al.*, 1999b), showed that the secondary structure of *rpoH* mRNA itself is a thermosensor. Previously, it was proposed that a 5' region of *rpoH* mRNA forms a secondary structure which inhibits ribosome binding at 37°C. Stress conditions, such as heat shock, would lead to the disruption of secondary structure, ribosome binding and enhanced translation of *rpoH* message. This hypothesis is supported by extensive experimental data and by the fact that the predicted secondary structure is conserved among many species of bacteria (Yuzawa *et al.*, 1993; 1995; Morita *et al.*, 1999a). Although at present it is believed that the intrinsic stability of *rpoH* mRNA secondary structure regulates heat shock response in bacteria, it not clear whether other RNA or



**Figure 1.** Schematic model showing a negative feedback control of the heat shock response in *E.coli* exerted by heat shock proteins (DnaK, DnaJ, GrpE and HflB). Upot heat shock,  $\sigma^{32}$  accumulates and regulates the expression of the heat shock proteins through its association with RNA polymerase (E). Heat shock proteins feedback negatively on  $\sigma^{32}$  at three levels: synthesis, stability and activity of  $\sigma^{32}$ .

protein factors are involved. A similar mechanism has also been proposed for other mRNAs which can function as physiological sensors of high or low temperature (Hoe and Gougen, 1993; Altuvia *et al.*, 1989). It is however interesting that this regulatory pathway mediated by  $\sigma^{32}$  may not be utilized for heat shock response in all bacteria.

## **Regulation of the heat shock response in Eukaryotes**

In Eukaryotes, expression of hsp is under control of a sequence-specific regulatory protein, heat shock transcription factor (HSF). HSF acts through a regulatory region of heat shock genes termed heat shock element (HSE). The first functional analysis of the regulatory region of the *Drosophila* hsp70 gene was done by examining the expression of a series of deletion mutants using a reporter gene in transiently transfected mammalian cells (Pelham, 1982). These experiments have identified sequences at position -66 to -47 necessary for heat-induced expression and defined a 14-bp consensus sequence (5'-CnGAAnnTTCnnG-3', where n can be any nucleotide), termed HSE, that was found in the promoters of all known hsp genes in *Drosophila*. When linked to the promoter of a heterologous reporter gene, HSE was able to confer heat inducible expression, providing definitive evidence of a unique regulatory element. In contrast, the basal promoter activity of hsp genes in higher Eukaryotes is HSE independent and is mediated by other sequence-specific

DNA-binding proteins (Abravaya *et al.*, 1991). Subsequent studies on the *Drosophila* hsp70 genes have revealed the importance of specific DNA sequences in and around consensus HSE (Amin *et al.*, 1988; Xiao and Lis, 1988) and led to a reevaluation of the HSE structure. All HSEs were found to be composed of contiguous inverted repeats of a 5-base pair (bp) sequence, 5'-nGAAn-3'. Sequence analysis of many hsp genes from *Drosophila* and mammals have revealed that although the number of HSEs as well as the number of the pentanucleotide units in an HSE can vary, the functional HSE is composed of a minimum of three 5-bp units. For example, HSEs from different *Drosophila* hsp genes were shown to contain from three to seven or eight 5-bp units, depending on the stringency used to define the 5-bp unit (Glaser *et al.*, 1990). The nucleotide sequence of the HSE is highly conserved from yeast to humans. In particular the guanine residue in the second position of each pentamer (5'-nGAAn-3') is absolutely conserved. The adjacent adenine residues are also highly conserved, but may be substituted by other bases. In the studies where the activity and interdependence of the HSEs in the context of the basal hsp promoter have been examined, it was shown that the HSE was rotationally independent from the basal promoter elements. Characterization of the promoter region of hsp genes in Eukaryotes that led to identification of the HSE, was the first step in the understanding of hsp gene regulation. When the chromatin structure of *Drosophila* hsp70 gene was analyzed *in vivo*, DNase I hypersensitive sites were detected in the 5'

flanking region (Wu, 1980). It was proposed that such sites might function as elements to which regulatory molecules can bind. Subsequent experiments using Exonuclease III footprinting indeed demonstrated the presence of constitutively bound factors to TATA box and factors whose binding to the HSE was induced by heat shock (Wu, 1984). Evidence for an activator of hsp genes also came from *in vitro* transcription and footprint analysis of *Drosophila hsp70* gene using nuclear extracts derived from heat-shocked *Drosophila* tissue culture cells (Parker and Topol, 1984). This binding activity was termed heat shock transcription factor (HSF) and was first purified from bulk quantities of *Saccharomyces cerevisiae*, *Drosophila* and human tissue culture cells, using HSE-affinity chromatography (Sorger and Pelham, 1987 (b); Wu *et al.*, 1987; Goldenberg *et al.*, 1988). As estimated from SDS-PAGE analysis, HSF was found to have different molecular sizes in each species (150 kD, 110 kD and 80 kD respectively).

## Cloning and characterization of HSFs

After the purification of HSFs, different strategies were used to clone HSF genes. In one, monoclonal antibodies directed against purified yeast HSF were used for cloning of a single copy *Saccharomyces cerevisiae* HSF gene by screening yeast cDNA expression libraries (Sorger and Pelham, 1988; Weiderecht *et al.*, 1988). In another approach, degenerate oligonucleotide probes, derived from peptide microsequencing, were employed for screening of a genomic

library and cloning of the single-copy *Drosophila* HSF gene (Clos *et al.*, 1990). Scharf and colleagues (Scharf *et al.*, 1990) used screening of cDNA expression libraries with HSE probes to clone three related HSF genes from tomato. Their results provided the first evidence that multiple members of the HSF family exist in an organism. The isolation of two related genes in human, HSF1 (Rabindran *et al.*, 1991) and HSF2 (Schuetz *et al.*, 1991) followed these findings. Human HSF1 was cloned using degenerated oligonucleotide probes and a similar approach was employed in cloning of two related HSF genes in mouse (Sarge *et al.*, 1991), three from chicken (Nakai and Morimoto, 1993) and one so far from *Xenopus* (Stump *et al.*, 1995). Recently, a new member of the HSF family, HSF4, has been cloned from human cells (Nakai *et al.*, 1997). Although vertebrates express several members of HSF family (HSF 1-4), studies showed that only HSF1 can respond to heat and other environmental and physiological stresses (Baler *et al.*, 1993; Sarge *et al.*, 1993; Fiorenza *et al.*, 1994). Therefore, HSF1 is the functional homologue of the general HSF. The only other heat-responsive HSF was cloned from chicken (HSF3); however its mode of activation differs from the one described for HSF1 (Nakai and Morimoto, 1995; Nakai *et al.*, 1995). Instead, mammalian HSF2 and HSF4 are refractory to typical stress stimuli. Rather, sequence-specific DNA binding of HSF2 is induced in certain developmental situations. For example, HSF2 is active during hemin-induced erythroid differentiation of human K562 cells (Sistonen *et al.*, 1992) and also in spermatogenetic cells of the mouse testis (Sarge *et al.*, 1994;



Fawcett *et al.*, 1994; Fiorenza *et al.*, 1995). In addition, HSF2 is constitutively active in mouse embryonic carcinoma cells and during early development of the mouse embryo (Metzger *et al.*, 1989, 1994; Murphy *et al.*, 1994). Little is known about HSF4 function yet (Nakai *et al.*, 1997). In plants, HSFs are also encoded by a multigene family (Scharf *et al.*, 1990; Czarnecka-Verner *et al.*, 1995). However, unlike vertebrate HSFs, some of the plant HSFs are heat shock inducible (Lyck *et al.*, 1997).

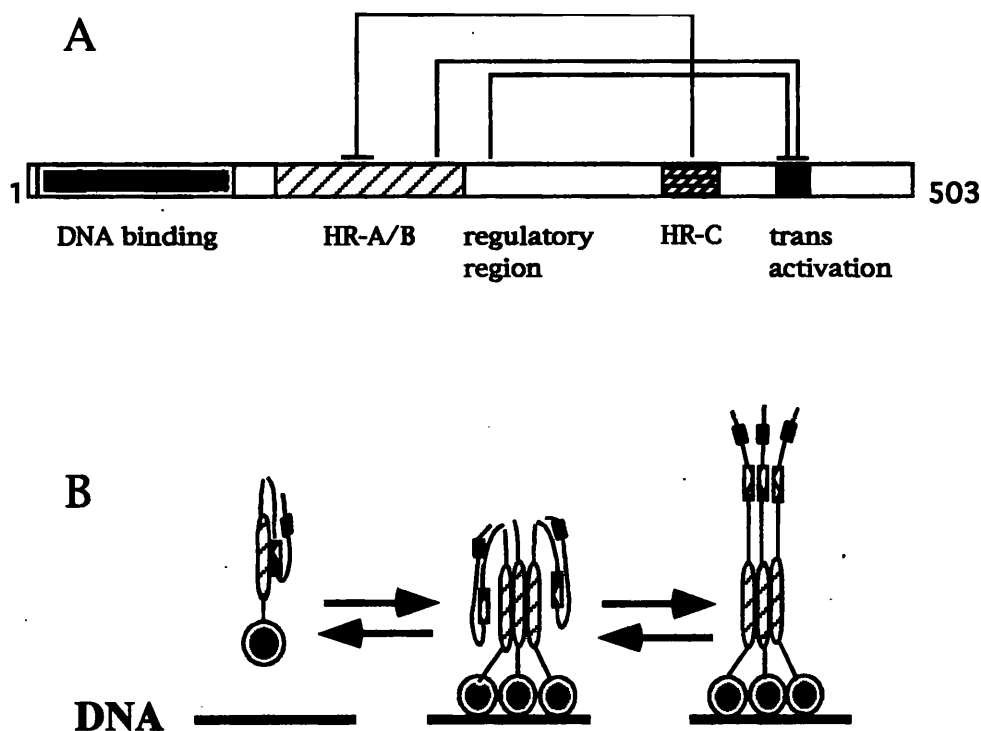
Additional complexity of the mammalian HSFs is generated by expression of different isoforms of HSF1, HSF2 and HSF4 that arise via alternative splicing (Fiorenza *et al.*, 1995; Nakai *et al.*, 1997). Since the level of both HSF1 and HSF2 and the relative abundance of their isoforms vary among tissues, this suggests their potential role in tissue-dependent regulation of the cellular stress response (Fiorenza *et al.*, 1995; Goodson and Sarge, 1995b).

Comparison of structure and sequences of the vertebrate HSFs revealed that within a single species, HSFs are 40% related in amino acid sequences (due to the extensive identity of DNA-binding and oligomerization domains, see below). Interspecies comparisons of HSF1 (between human, mouse and chicken) indicate 80-95% of sequence conservation. Multiple HSFs in vertebrates and plants probably arose from a need of larger and complex organisms to respond to a diverse array of developmental and environmental cues by co-regulating hsp genes in response to distinct signals. Considering the data demonstrating synergistic transcriptional activation of hsp genes by two

HSFs (HSF1 and HSF2) activated by two different stimuli (heat shock and hemin) in human erythroleukemia K562 cells, (Sistonen *et al.*, 1992), this assumption seems reasonable.

## Molecular organization of HSF

The cloning of the HSFs allowed interspecies comparison of the deduced amino acid sequence. This analysis revealed two major regions of local amino acid conservation in the amino terminal part, conserved in all known HSFs. By deletion analysis of yeast and *Drosophila* HSF, they were initially found to function in the specific and high affinity binding to HSEs (Weiderrecht *et al.*, 1988; Clos *et al.*, 1990) and the same was later confirmed in experiments with mammalian HSFs. Figure 2A shows molecular organization of conserved domains in mouse HSF1. The first conserved region is the DNA-binding domain, the most conserved region. The DNA-binding domain of HSFs did not appear to belong to any known category of DNA-binding motifs, except two pentapeptide sequences that are highly similar to bacterial  $\sigma$ -factors (Clos *et al.*, 1990) and a short region with a more limited similarity to the HNF3/*forkhead* DNA-binding motif (Scharf *et al.*, 1994). However, when the crystal structure of *Klyveromyces lactis* and the solution structure of *K. lactis* and *Drosophila* HSF DNA-binding domains became available, they revealed that the DNA-binding domain of HSF belongs to the well-known family of "winged" helix-turn-helix DNA-binding motifs (Hubl *et al.*, 1994; Torres *et al.*, 1995). These two pieces of data were



**Figure 2.** (A) Schematic presentation of structural domains of HSF1. The DNA-binding domain (amino acids 16-120) is localized to the amino terminus (gray box). Adjacent to it is the trimerization domain, HR-A/B (hatched box, amino acids 137-212). The carboxy terminus contains a leucine zipper, HR-C (cross-hatched box, amino acids 378-407), involved in suppression of trimerization and transcriptional activation domain (black box), which is negatively regulated by the residues within the trimerization domain and by the regulatory region located immediately adjacent to the trimerization domain. (B) A possible model for HSF1 regulation in higher Eukaryotes. In the absence of stress, HSF1 is maintained as an inactive monomer through intramolecular coiled-coil (left). Activation of HSF1 is thought to occur in two (reversible) steps: 1. stress-induced trimerization (through intermolecular coiled-coil) required for sequence-specific DNA-binding (middle) 2. exposure of transactivation domains in the trimer enables acquisition of full transcriptional competence (right). The figure is freely redrawn from Wu, 1995.

consistent and demonstrated the presence of three  $\alpha$ -helices and a small four-stranded, anti-parallel  $\beta$ -sheet in the DNA-binding domain. Biochemical and genetic experiments with yeast HSF showed that the second helix of the helix-turn-helix motif was the recognition helix.

The second region conserved among all HSFs has been shown to be required for the oligomerization (Sorger and Nelson, 1989; Clos *et al.*, 1990) (Figure 2A). This region (heptad repeats A and B, HR-A/B) is located adjacent to the DNA-binding domain and contains three arrays of hydrophobic heptad repeats. Hydrophobic residues at the first and the fourth positions of a heptad repeat are characteristic of  $\alpha$ -helical coiled-coil structures (Cohen and Parry, 1990, 1994). Leucine zippers are found in many DNA-binding proteins that typically associate as homo- or heterodimers through  $\alpha$ -helical coiled-coil interaction. The number and arrangement of the heptad repeats, (termed leucine zippers 1-3), in the oligomerization domain of HSFs is however unusual, since the first, long hydrophobic array consist of five or six repeats, while the remaining two short arrays overlap and are positioned one residue out of phase. The unusual number of leucine zipper motifs in HSFs suggests that these proteins can oligomerize to states more complex than the dimer. This was found to be the case when the oligomerization state of yeast and *Drosophila* was assayed. By coexpressing *in vitro* truncated forms of yeast HSF and analyzing the number of heteromeric species in the gel mobility shift assay with the HSE, Sorger and Nelson (1989)

found that HSF binds to the HSE as a trimer. In addition, in the same report, they demonstrated, using chemical cross-linking, that yeast HSF also forms trimers in solution. Similarly, chemical cross-linking of purified HSF, either natural or cloned, was employed to demonstrate that the oligomerization state of the active DNA-binding form of *Drosophila* HSF is primarily trimeric (Perisic *et al.*, 1989) or hexameric (Clos *et al.*, 1990). Other studies done with HSFs from different species using either chemical cross-linking or hydrodynamic analysis confirmed these findings (Baler *et al.*, 1993; Sarge *et al.*, 1993; Rabindran *et al.*, 1993; Westwood and Wu, 1993). Earlier, Sorger and Nelson (1989) also proposed that trimerization occurs through a three stranded coiled-coil structure. In this structure, the first array of repeats forms a long  $\alpha$ -helix, which mediates the interaction between HSF monomers constituting a major part of the HSF trimer interface. This interaction occurs through a hydrophobic surface formed by amino acids (commonly leucine, isoleucine or valine) at every first and third or fourth position within a heptad repeat. In addition, the second and third repeats also form a small  $\alpha$ -helix, which would contribute to the stability of interaction between longer helices. Biophysical studies on yeast HSF also suggested a similar model of interaction between trimerization domains (Peteranderl and Nelson, 1992), although the physical structure for this domain has not yet been determined. Although the residues at the first and the fourth positions of the heptad repeat are generally conserved among HSFs, only two such residues are invariant

among HSFs cloned so far. Multiple sequence alignments also revealed variations in other positions of the heptad repeat. For example, the second and third short arrays of heptad repeats are interrupted by a small insertion for the plant HSFs. These differences are thought to confer specificity of the interaction between trimerization domains since no mixed oligomers can be detected when different HSFs are coexpressed in the same cell (Clos *et al.*, 1993; Rabindran *et al.*, 1993; Sistonen *et al.*, 1994).

## HSF-HSE Interaction

When the interaction between HSF and HSE was studied, it was found that HSF binds to the HSE as a trimer and that all three pentanucleotide repeats of the HSE are required for high-affinity interaction (Sorger and Nelson, 1989; Perisic *et al.*, 1989). Using the high-resolution chemical and DNase I footprinting assays, Perisic and colleagues (Perisic *et al.*, 1989) tested *in vitro* binding of a purified recombinant *Drosophila* HSF to HSEs with different numbers and arrangements of pentanucleotide units. The smallest array showing detectable binding to HSF is two-repeat HSE to which HSF binds with a moderate affinity. These authors also demonstrated that HSF binds with a similar affinity to such an incomplete HSE regardless of the orientation of pentanucleotide units (nGAAnnTTcn and nTTCnnGAAn, which are referred to as head-to-head and tail-to-tail repeats, respectively). Therefore, the nGAAn is considered to be a

fundamental unit of recognition, with each subunit of the HSF trimer interacting with one of three nGAAn repeats within the complete HSE which contains three pentanucleotide units that constitute the minimal number for a high-affinity interaction. This was later confirmed by experiments in which 1:1 stoichiometry for the binding of a recombinant DNA-binding domain of *Drosophila* HSF to an nGAAn site was observed (Kim *et al.*, 1994). These findings were followed with the observation that multiple HSFs bind cooperatively to HSEs with four or more pentanucleotide repeats and that this cooperative binding shows a strong thermal dependence (Xiao *et al.*, 1991). Earlier, evidence of cooperative protein-protein interactions between two HSF trimers, came from *in vitro* study in which it was found that the affinity of *Drosophila* HSF for an HSE from the hsp70 promoter (which contains four HSEs) is much higher when the adjacent HSE is present than when it is deleted (Topol *et al.*, 1985). These data, together with the findings of other groups working on yeast or vertebrate HSFs (Bonner *et al.*, 1994; Drees *et al.*, 1997; Kroeger and Morimoto, 1994) indicated that HSF makes cooperative interactions both between subunits within an HSF trimer and between adjacent trimers. Moreover, cooperative binding of bacterially expressed HSF trimers to adjacent HSEs was visualized by electron microscopy as a protein-induced DNA loop (Wyman *et al.*, 1995). Recently, it was shown that the residues from the DNA-binding domain of *K. lactis* HSF are involved in the cooperative interaction between adjacent trimers through

protein-protein contacts (Littlefield and Nelson, 1999). The same residues are likely to be important for increasing the DNA-binding specificity and affinity of trimeric HSF for the HSE. Since HSEs from different hsp genes differ in the number of pentanucleotide repeats, the conservation of repeats to the nGAAn consensus and the distance between HSE and the basal promoter elements, this may allow HSF to control different levels of stress-induced expression of the various hsps (Xiao *et al.*, 1991).

## Oligomerization of HSF

A prominent regulatory feature of HSF is oligomerization to an active DNA-binding state. HSF from *Drosophila*, human and mouse are constitutively synthesized and maintained in the cell in a latent, monomeric state under the normal conditions of growth. Upon heat shock, they are converted into a DNA-binding competent trimer (Westwood *et al.*, 1991; Westwood and Wu, 1993; Baler *et al.*, 1993; Sarge *et al.*, 1993; Zuo *et al.*, 1994; Zandi *et al.*, 1997). The biochemical events associated with the oligomeric transition have been studied by means of different biochemical assays such as electrophoresis on native limiting-pore-size gels, gel filtration chromatography, chemical crosslinking and hydrodynamic studies. These experiments yielded consistent results with respect to the change in oligomerization of HSF on heat treatment i.e. monomer to trimer transition. Heat shock induced trimerization increases the affinity of HSF for



binding to the HSE (Wu, 1985; Zimarino and Wu, 1987). This was further demonstrated using a semi-quantitative filter-binding assay as well as *in vitro* footprinting studies with HSF trimers purified from heat shocked cells (Wu *et al.*, 1987; Taylor *et al.*, 1991). Consistently, genomic footprinting analysis revealed that the HSEs of the human hsp70 promoter are occupied *in vivo* only after heat shock, but not prior to or after recovery from heat stress (Abravaya *et al.*, 1991). In *Drosophila*, heat-induced activation of HSF is accompanied by a rapid accumulation of HSF on the heat shock puffs of the polytene chromosomes (Westwood and Wu, 1991). Taken together, these studies suggest that the conversion of HSF from its inactive, monomeric form to the active, DNA-binding competent trimer is subjected to stringent control, presumably because the level of hsps has to be tightly controlled. Indeed, overexpressing hsp70 under the normal conditions in *Drosophila* cells can inhibit cell growth (Feder *et al.*, 1992).

A central question has been to understand how trimerization of HSF is suppressed under normal environmental conditions. Some data indicated an involvement of a region (heptad repeat C, HR-C) located near the carboxy-terminal end of HSF (Figure 2A). This region (known as leucine zipper 4) contains another array of hydrophobic heptad repeats and is well-conserved among vertebrate HSFs, but poorly conserved in plant and yeast HSFs. Since both non-conservative substitutions of hydrophobic residues in or deletion of the leucine zipper 4 resulted in

activation of HSF1, leucine zipper 4 was proposed to be involved in suppression of oligomerization (Rabindran *et al.*, 1993; Zuo *et al.*, 1994; Zuo *et al.*, 1995; Nakai and Morimoto, 1993; Figure 2A). Similarly, mutations in both long and short array of heptad repeats in the trimerization domain also led to constitutive oligomerization. Zuo and colleagues (Zuo *et al.*, 1994) have found through analysis of a set of human HSF1 mutants expressed in *Xenopus* oocytes, that small deletions or non-conservative substitutions of hydrophobic residues in heptad repeats of the trimerization domain (leucine zippers 1-3) led to constitutive trimerization and DNA-binding ability. The results of the mutagenesis experiments suggested that both amino- and carboxy-terminal heptad repeats participate in the maintenance of an inactive state of HSF (Rabindran *et al.*, 1993; Zuo *et al.*, 1994). This most probably occurs through intramolecular coiled-coil interaction between leucine zippers, although such an interaction has yet to be directly demonstrated. This model (Figure 2B) predicts that the inert HSF monomer is constrained by intramolecular coiled-coil interactions under physiological conditions. Upon heat stress, such an interaction is disrupted and HSF is converted to DNA-binding competent trimer. The trimer forms by intermolecular coiled-coil interaction between leucine zippers of the trimerization domains resulting in a triple-stranded coiled-coil. This transition is however reversible; during the recovery from the stress conditions, HSF trimers would dissociate to monomers. Evidence from work in which properties of the trimerization domain of yeast HSF were

examined, (Peteranderl and Nelson, 1992), suggest formation of a triple-stranded coiled-coil. In plants, amino- and carboxy-terminal heptad repeats also seem to be involved in trimer regulation, as inferred from experiments with tomato HSFA1 (Scharf *et al.*, 1990). On the contrary, substitutions of the hydrophobic residues in the poorly conserved carboxy-terminal heptad repeats of yeast HSF, did not have any effect on HSF function (Chen *et al.*, 1993) and their role remains unclear.

Trimerization domain and DNA-binding domain are connected by the linker region defined as the residues that separate the end of the DNA-binding domain from the start of the trimerization domain. Its length varies among HSFs and in yeast *S. cerevisiae* a substantial portion of the linker region can be deleted without affecting function (Flick *et al.*, 1994). However, a recent report showed that a small region in the amino-terminal portion of the human HSF1 linker is required *in vivo* for maintenance of HSF1 in monomeric state, thus contributing to the regulation of HSF1 monomer-trimer equilibrium (Liu and Thiele, 1999).

## **Transcriptional activation function of HSF**

Sequence alignments of HSFs did not reveal amino acid conservations other than the DNA-binding, trimerization domain and carboxy-terminal heptad repeat. A domain responsible for transcriptional activity (transactivation domain) was identified in yeast HSF. By fusing pieces of yeast

HSF to a heterologous DNA-binding domain and testing the chimeras in a transcriptional assay, sequences important for transcriptional activity have been mapped to amino- and carboxy-terminal regions (Nieto-Sotelo *et al.*, 1990; Sorger, 1990; Jacobsen and Pelham, 1991; Bonner *et al.*, 1992; Chen *et al.*, 1993). The amino-terminal activator in *S. cerevisiae* functions during sustained stress, whereas the carboxy-terminal transactivator responds to transient stress. Similarly, the domains involved in transcriptional activity of tomato, *Drosophila* and vertebrate HSFs have been studied through deletion mapping and transcriptional assays in the context of GAL4/HSF chimeras. These experiments revealed that the transactivation domain is located in the carboxy-terminal portion (Figure 2A) (Treuter *et al.*, 1993; Scharf *et al.*, 1994; Green *et al.*, 1995; Zuo *et al.*, 1995; Shi *et al.*, 1995; Newton *et al.*, 1996). In addition, they defined a minimal region for transcriptional activation in mammalian HSF1 which is rich in acidic and hydrophobic residues. Interestingly, in both yeast and vertebrate HSFs, the transactivation domain is restrained under normal growth conditions by different regions of the proteins. In yeast, the DNA-binding domain, the trimerization domain and a short conserved element, CE2, located between the trimerization domain and the transactivation domain, negatively regulate the transactivation domain (Nieto-Sotelo *et al.*, 1990; Jacobsen and Pelham, 1991; Bonner *et al.*, 1992). Amino acid substitutions in these regions led to increased transcriptional activity of HSF suggesting that besides their role in sequence-specific DNA binding and oligomerization,

the conserved domains are also involved in the suppression of the activator domain under basal conditions. Similarly, the transcriptional competence of human HSF1 is negatively regulated during normal growth conditions. Single-residue substitutions or deletions in the leucine zipper 2 of the trimerization domain and nearby downstream sequence (termed regulatory domain, Figure 2A) increased the ability of human HSF1 to activate the reporter gene in *Xenopus* oocyte (Zuo *et al.*, 1995). Based on these findings, it appears that the transcriptional competence of mammalian HSF1 is regulated by the trimerization domain and the regulatory domain (Figure 2A). Importantly, these experiments provided the evidence that the trimerization region plays a role in the negative regulation of the transcriptional activity of HSF1 that is independent from its involvement in the control of the oligomeric state of the factor. This is most probably achieved through intramolecular interactions in an inactive, monomeric HSF1.

### **The role of phosphorylation in HSF1 functioning**

Despite the difference in regulation, both yeast and higher Eukaryotic HSFs were found to exhibit stress-induced phosphorylation. This was first described for yeast HSF where stress-induced phosphorylation results in a retarded electrophoretic mobility of HSF polypeptides on SDS-PAGE that is reversed by phosphatase treatment (Sorger *et al.*, 1987;

Sorger and Pelham, 1988). Similar observations have been made for mammalian HSF1 (Larson *et al.*, 1988; Sarge *et al.*, 1993). These results were extended by direct  $^{32}\text{P}$ -labeling experiments, which demonstrated increased phosphorylation at serine and threonine residues upon heat shock for both yeast HSF and mammalian HSF1 (Sorger, 1990; Rabindran *et al.*, 1994), although the positions of these serine and threonine residues have not been mapped. Initial experiments demonstrated that inducible phosphorylation of yeast HSF correlates with transcriptional activity; however, other data suggest that the hyperphosphorylation of serine residues adjacent to a conserved element (CE2) in the carboxy-terminus of yeast *K. Lactis* HSF may be important in the deactivation of the factor (Hoj and Jacobsen, 1994). Therefore, the available data addressing a causal relationship between phosphorylation and the activation or deactivation of yeast HSF are inconclusive and it can be envisaged that phosphorylation can have both positive and negative effects on the activity of HSF.

Several groups have studied stress-induced hyperphosphorylation of mammalian HSF1. The experiments in which non-steroidal anti-inflammatory drugs (such as salicylate or indomethacin) were used to activate *in vivo* HSF1 have proven useful in determining the role of inducible phosphorylation in the activation of HSF1 (Jurivich *et al.*, 1992; Sarge *et al.*, 1993; Cotto *et al.*, 1996). Treatment of mammalian cells with these drugs induces HSF1 DNA-binding activity; however, the drug-induced form of HSF1 is

transcriptionally inert. This inert intermediate can be converted to the transcriptionally active state by a subsequent exposure to heat shock. Similarly, exogenous HSF1 overexpressed in human cells that is trimeric but transcriptionally inert can be activated by treatment of transfected cells with calyculin A (an inhibitor of serine/threonine protein phosphatases) (Xia *et al.*, 1997). This led to a suggestion that the stress-induced activation of HSF1 may be a multistep process (Figure 2B) in which trimerization and acquisition of DNA-binding ability is necessary but insufficient for transcriptional activation. An additional step is required for the full transcriptional competence of HSF1 and the acquisition of transcriptional activity is linked to inducible hyperphosphorylation. The transcriptionally inert HSF1 trimer, resulting from HSF1 overexpression, may correspond to the constitutive DNA-binding activity exhibited by *Saccharomyces cerevisiae* HSF (Sorger and Pelham, 1988). However, in other studies it was demonstrated that the treatment of mammalian cells with an amino acid analogue, L-azetidine-2-carboxylic acid, induces DNA-binding activity of HSF1. This form of HSF1 is also transcriptionally active, although it is not hyperphosphorylated (Mosser *et al.*, 1988; Williams and Morimoto, 1990; Sarge *et al.*, 1993), suggesting that hyperphosphorylation of HSF1 is not essential for its trimerization, DNA-binding and transcriptional activities. Xia and Voellmy also addressed the role of stress-induced phosphorylation of human HSF1 (Xia and Voellmy, 1997). By combining the treatment of cultured cells with heat

shock and serine/threonine protein kinase inhibitors (H7 and GF-X) or protein phosphatase inhibitor calyculin A, they demonstrated that stress-induced hyperphosphorylation both stimulates the transactivation function of HSF1 and also prolongs trimerization and activity subsequent to heat shock. Since all these effects may also be related to the changes in the balance of kinase and phosphatase activities that are known to occur during heat stress, at present, the functional role of inducible hyperphosphorylation of HSF1 remains unclear.

In Metazoa, inactive HSF1 was also found to be basally phosphorylated on serines and this phosphorylation negatively regulates transcriptional activity at physiological temperature (Knauf *et al.*, 1996; Chu *et al.*, 1996; Kline and Morimoto, 1997; Chu *et al.*, 1998; Dai *et al.*, 2000). This was shown using different approaches. Mivechi and Giaccia found that overexpression of a dominant inhibitory mutant of mitogen activated protein kinase (MAPK) ERK1 increases the expression of an hsp70 promoter-driven reporter gene in NIH 3T3 cells (Mivechi and Giaccia, 1995). Others have shown that expression of activated Raf kinase in human 293 cells results in increased phosphorylation of an epitope-tagged exogenous HSF1 (Knauf *et al.*, 1996). It was also demonstrated by cotransfection of HSF1 and MAPKK (MEK1) in NIH3T3 cells with an hsp70 promoter-driven reporter gene, that overexpression of MEK1 reduces reporter gene expression (Chu *et al.*, 1996). Taken together, these data suggested that, under conditions when the MAP kinase pathway is activated, HSF1



transcriptional activity is inhibited. Further experiments using phosphopeptide mapping of GAL4-human HSF1 chimeras, or recombinant HSF1 phosphorylated *in vitro*, concluded that HSF1 is constitutively phosphorylated on serines 303, 307 and 363. Transactivation assays suggested an important role for serines 303 and 307 in repressing the transcriptional ability of the factor. Substitution of these residues by alanines, resulted in loss of suppression of transactivation without affecting trimerization and DNA-binding of transiently transfected mutant HSF1 (Knauf *et al.*, 1996; Chu *et al.*, 1996; Kline and Morimoto, 1997). Importantly, phosphorylated serines 303 and 307 are located in the regulatory domain which, as mentioned above, was previously identified to suppress transcriptional activity of HSF1 and also to confer heat responsiveness to an otherwise heterologous chimeric activator (Zuo *et al.*, 1995; Newton *et al.*, 1996). The identity of protein kinases responsible for constitutive phosphorylation of HSF1 has also been addressed. Sequence comparisons revealed that the immediate and flanking sequences surrounding serines 303 and 307 are highly conserved in vertebrate HSF1s and correspond to consensus sequence for proline-directed kinases including members of mitogen-activated kinases (MAPKs). Chu and colleagues (Chu *et al.*, 1998) reported that the constitutive phosphorylation of human HSF1 appears to be hierarchical: mitogen-activated protein kinases of the ERK1 family phosphorylate HSF1 on serine 307, which primes HSF1 for subsequent phosphorylation on serine 303 by glycogen

synthase kinase 3 $\beta$  (GSK3 $\beta$ ). In the same report, they demonstrated that the serine at position 363 is phosphorylated *in vitro* by protein kinase C isoforms,  $\alpha$  and  $\xi$ . This finding was questioned recently by a report showing that serine 363 is phosphorylated *in vitro* by a member of another family of MAP kinases, c-Jun terminal kinase (JNK) (Dai *et al.*, 2000). Dai and colleagues also presented evidence, using an immunoprecipitation assay that HSF1 interacts *in vivo* with both ERK1 and JNK. In addition, similar to ERK1, JNK overexpression also suppresses transcriptional activity of HSF1.

In summary, HSF1 seems to be regulated by the action of several protein kinase cascades and this may ensure suppression of its transactivation function at 37°C and during recovery from stress. However, it remains to be determined which kinases actually phosphorylate HSF1 *in vivo*. In addition, it is also unclear how different signaling pathways can cooperate in the suppression of the transactivation function of HSF1 since they are activated by diverse stimuli.

## Regulation of HSF1 activation

There is a fundamental difference between the regulation of HSF activity in the budding yeast *Saccharomyces cerevisiae* and the activity of its functional counterpart, HSF in *Drosophila* or HSF1 in vertebrates. Yeast HSF is constitutively trimeric and bound to DNA *in vivo*; also, a significant level of HSE binding activity can be detected *in*

*vitro* at non heat shock temperatures (25°C) (Sorger *et al.*, 1987 (b); Szent-Gyorgyi *et al.*, 1987; Jacobsen and Pelham, 1988). Heat shock does not lead to a further increase in DNA-binding activity of yeast HSF, but is required for the transcriptional activity. In contrast, metazoan HSF1 is strictly negatively regulated and heat shock responsive (Kingston *et al.*, 1987; Zimarino and Wu, 1987; Mosser *et al.*, 1988). Interestingly, HSF from the fission yeast *Schizosaccharomyces pombe* exhibits the mode of regulation similar to the one found in higher Eukaryotes, since heat shock is required for both DNA binding and transcriptional activity (Gallo *et al.*, 1991). Initial experiments demonstrated the existence of an HSE binding activity in the crude extracts of *Drosophila*, *Xenopus* and mammalian cells (Kingston *et al.*, 1987; Zimarino and Wu, 1987; Zimarino *et al.*, 1990) using a gel mobility shift assay. This binding activity, later found to be HSF, is present in a latent state and is induced by heat shock. Using the gel mobility shift assay with a consensus HSE, the presence of active HSF can be detected in extracts of heat shocked cells within minutes after heat shock treatment. The increase in HSF binding to the HSE is correlated with the severity of the heat stress, suggesting the temperature-dependent modulation of HSF-binding activity as a critical regulatory switch in the activation of hsp gene transcription. The induction occurs even in the presence of a protein synthesis inhibitor, cycloheximide, indicating that it is regulated post-translationally. In this experiment, it was demonstrated that HSF DNA-binding activity in *Drosophila* cells can be

sequentially induced through several cycles of heat shock and relaxation in the continuous presence of cycloheximide (Zimarino and Wu, 1987). Similar results were also obtained with HSF1 in human cells (Kingston *et al.*, 1987). These data are in agreement with earlier studies in which it was found that the induction of heat shock puffs on the polytene chromosomes of *Drosophila* was independent of protein synthesis (Ashburner, 1970).

The effect of heat stress on activation of HSF *in vivo* can be mimicked *in vitro*. If cytosolic extracts of unshocked *Drosophila* or mammalian cells are incubated at heat shock temperature, HSF is activated and binds to HSE. HSF trimerization and DNA-binding *in vitro* can also be induced in crude extracts by a variety of treatments that affect protein structure such as mild detergents, low or high pH, urea or anti-HSF antibodies (Larson *et al.*, 1988; Mosser *et al.*, 1990; Zimarino *et al.*, 1990; Zimarino *et al.*, 1990). A step forward in evaluation of the ability of HSF to respond directly to environmental stress came from the experiments in which purified inactive HSF1 was tested. These studies showed that *in vitro* activation by heat shock can be reproduced with a purified recombinant human and mouse HSF1 monomer, expressed in either rabbit reticulocyte lysate or in *E. coli* (Larson *et al.*, 1995; Goodson and Sarge, 1995a; Farkas *et al.*, 1998). However, the temperature-sensitive range did not match inducing temperatures *in vivo*. Importantly, the results from Farkas and colleagues suggested that intramolecular repression was a central mechanism for the regulation of HSF1

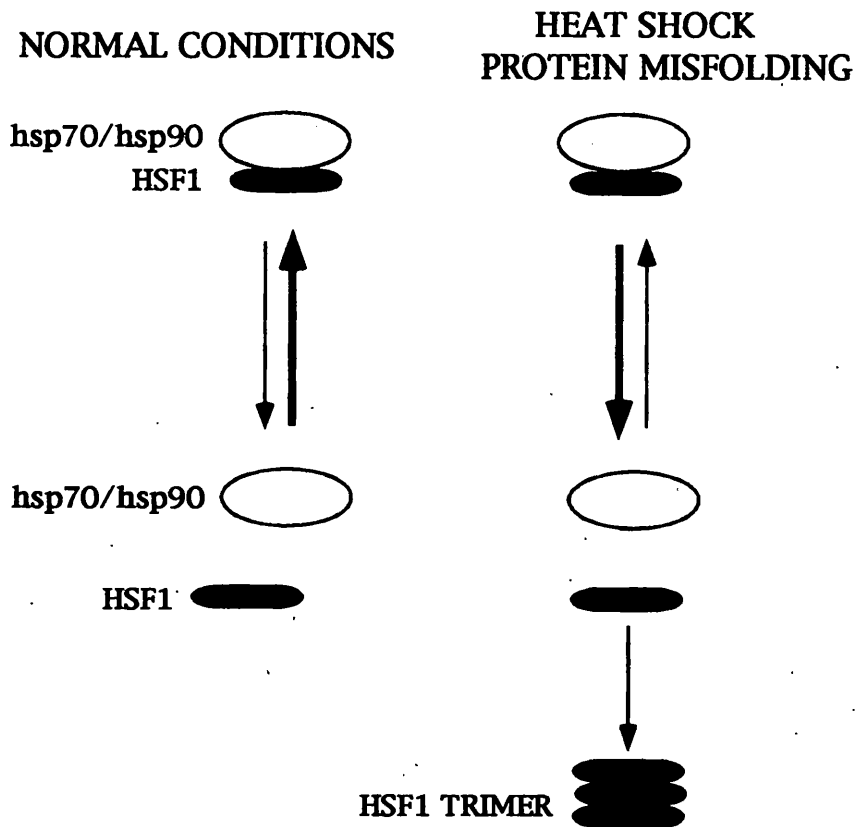
activity, because the repression of purified, inactive HSF1 monomer could be relieved in the apparent absence of regulatory proteins. Zhong and colleagues reported that trimerization and DNA-binding of purified *Drosophila* HSF can be directly and reversibly induced *in vitro* by heat shock and by an oxidant, hydrogen peroxide (Zhong *et al.*, 1998). Their data for the first time clearly demonstrated that HSF is directly and reversibly capable of sensing elevated temperatures in the physiological range and of transducing the thermal signal to a change in trimer association and DNA-binding activity.

The results of *in vitro* experiments with purified recombinant factor suggest that the elements residing in the *Drosophila* HSF or mammalian HSF1 polypeptides are sufficient for both monomer maintenance and for its heat-induced conversion to the trimeric, DNA-binding form. This is in support of a role for eukaryotic HSF as a "molecular thermometer" which directly senses environmental conditions and activates cellular stress response. However, these intrinsic properties of HSF may be modulated *in vivo* by cellular factors and/or covalent modifications. For example, the temperature at which HSF is activated is not strictly intrinsic to the factor, but can be reprogrammed according to the cellular environment in which HSF is expressed. When human HSF1 is expressed in insect, frog or plant cells, its induction temperature is reset to the heat shock temperature of the host cell (Clos *et al.*, 1993; Baler *et al.*, 1993; Treuter *et al.*, 1993). Conversely, when *Drosophila* HSF is expressed in human cells,

it acquired constitutive activity at ambient temperature of 37°C. In addition, the induction temperature for mouse HSF1 is reduced in testis, where the normal temperature is lower (Sarge *et al.*, 1995). Abravaya and colleagues also demonstrated that the growth of HeLa cells at suboptimal temperature (35°C), led to a corresponding decrease in the temperature required to activate HSF1 (40-41°C). This temperature (40-41°C) is normally insufficient for maximal activation when cells are grown at 37°C (Abravaya *et al.*, 1991). Taken together, these studies indicate the importance of the internal physiology of the cell, since the temperature at which HSF is activated is not absolute.

Other studies suggested that the generation and maintenance of the inactive HSF monomer might require participation of intracellular regulators present in limiting amounts that may exert their activity through suppression domains of HSF. Often, depending on the experimental conditions used, overexpression of HSF at 37°C in *E.coli* or in eukaryotic cells results in deregulation i.e. trimerization and constitutive DNA-binding (Clos *et al.*, 1990; Rabindran *et al.*, 1991; Sarge *et al.*, 1993). Among the many possible intracellular regulators of HSF, hsp's are candidates for controlling the activity of HSF. The first observation indicating a role for hsp's came from the experiments showing that reduced amounts of two constitutively expressed hsp70 genes in yeast *S. cerevisiae*, SSA1 and SSA2, led to increased transcriptional activity of HSF (Boorstein and Craig, 1990). Based on this, a model was proposed (Figure 3) in which heat

shock proteins, particularly hsp70, negatively control the activity of HSF under normal conditions. Upon heat shock, denatured and aggregated cellular proteins would titrate hsps from HSF and this sequestration of hsp70 and possibly of other chaperones releases HSF from its negatively regulated state. HSF trimerizes, binds to DNA and activates the synthesis of hsps that then feeds back to repress HSF activity when restored to appropriate levels. This mechanism for negative feed back control is similar to that proposed for  $\sigma^{32}$  in bacteria (Figure 1; Craig and Gross, 1991). Some data from experiments in *Drosophila* and *Xenopus laevis* argue in favour of such a model. First, a number of mutations in *Drosophila* induce tissue-specific activation of hsp genes *in vivo* in the absence of heat shock (Hiromi *et al.*, 1986; Parker-Thornburg and Bonner, 1987). Second, injection of denatured proteins into *Xenopus* oocytes resulted in the induction of hsp reporter gene or HSF DNA-binding activity (Ananthan *et al.*, 1986; Mifflin and Cohen, 1994). However, the experiments with hsp70 overexpression in mammalian cells yielded inconsistent results. In agreement with predictions, constitutive overexpression of hsp70 inhibited DNA-binding of human HSF1 after heat stress (Mosser *et al.*, 1993). There was a correlation between the level of inhibition and level of hsp70. The same effect was seen if hsp70 is transiently overexpressed in mammalian cells (Baler *et al.*, 1996). Moreover, the inhibitory effect of Hsp70 on HSF1 activation *in vitro* was reported (Abravaya *et al.*, 1992). In contrast, another group reported that there is no such inhibition *in*



**Figure 3.** A possible model of regulation of HSF1 by chaperones. Under normal conditions, most HSF1 molecules are present in a complex with hsp70 and/or hsp90. Heat shock or other stress conditions provoke accumulation of misfolded proteins which compete with HSF1 binding to hsp70/hsp90. As a result, concentration of free HSF1 increases and trimerization is favoured. Freely redrawn from Zou *et al.*, 1998.



*vivo*, both in *Drosophila* and mammalian cells (Rabindran *et al.*, 1994). However, they and others (Kim *et al.*, 1995) found that the kinetics of trimer dissociation is accelerated by hsp70 overexpression. On the other hand, the results of the experiments showing that DNA-binding activity of *Drosophila* and *Xenopus* HSF is capable of being sequentially induced by heat stress, deactivated, and reinduced in the absence of *de novo* protein synthesis, question a feedback inhibition by inducible hsps on HSF1 DNA-binding (Zimarino *et al.*, 1990). A direct interaction between HSF1 and hsp70 was detected in several biochemical assays. Anti-hsp70 antibody supershifted an HSE-HSF1 complex from heat shocked HeLa cells in a native gel mobility assay demonstrating stable *in vitro* association between activated HSF1 and hsp70 (Abravaya *et al.*, 1992). Notably, the HSF1/hsp70 complex was dissociated upon addition of ATP, a cofactor known to mediate the release of bound substrates from hsp70 (Freeman *et al.*, 1995). Similar observations have also been reported by others (Baler *et al.*, 1992; Baler *et al.*, 1996) using native PAGE and anti-hsp70 antibodies. Substoichiometric amounts of hsp70 were also captured in immune complexes with both inactive and active human HSF1 (Rabindran *et al.*, 1994), while Nunes and Calderwood (1995) found that HSF1 and hsp70 can be cross-linked in the extract of unstressed mouse cells. Others found that hsp70 binds to and represses the transcriptional activation domain of mouse HSF1, thereby acting during the attenuation phase of the heat shock response and perhaps having also a role in trimer disassembly (Shi *et al.*, 1998).

However, the above interactions appear not to significantly affect the DNA-binding ability of HSF1 and their functional significance remains to be determined. It is also unlikely that hsp70 acts alone in these processes as it is known that hsp70 is a component of multichaperone complexes involved in protein folding and assembly (Hartl *et al.*, 1992).

Hsp90 was also reported to interact with HSF1 in a hsp90 affinity chromatography experiment (Nadeau *et al.*, 1993). *In vitro* reconstitution assay have also revealed that hsp40, hsp90 and hsp90 multichaperone constituents hsp/c70, Hop, Hip, p23, Cyp40 and FKPBs, have a propensity for associating with recombinant active trimeric human HSF1 (Nair *et al.*, 1996; Voellmy, 1996). Furthermore, certain benzoquinone ansamycins, such as geldanamycin and herbimycin A, specifically bind hsp90 disrupting complexes with target proteins *in vitro* and *in vivo* (Whitesell *et al.*, 1994; Smith *et al.*, 1995). The same compounds also activate HSF1 *in vivo* (Hedge *et al.*, 1995; Zou *et al.*, 1998), raising the possibility that hsp90 may participate in negative regulation of HSF1. However, it is not clear whether induction of the heat shock response by geldanamycin and herbimycin A is directly related to their effect on hsp90, since both drugs are also potent redox-cyclers and may cause oxidative protein denaturation. In order to clarify the putative role of hsp90 in HSF1 regulation, Zou and colleagues developed an *in vitro* system to study HSF1 activation (Zou *et al.*, 1998). They found that HSF1 in extracts from nonstressed cells, besides heat shock, can be activated *in vitro* also by geldanamycin

and denatured proteins and that the addition of hsp90, but not hsp70, prevented trimerization of HSF1. Consistently, immunodepletion of hsp90, but not of hsp70, from the extract, resulted in HSF1 activation. Based on this and the finding that HSF1 can be *in situ* cross-linked in a complex with hsp90, they proposed that hsp90 is a major repressor of HSF1 through a dynamic complex that exists under physiological conditions and rapidly dissociates during heat stress (Figure 3). It remains unclear whether hsp90 acts as a repressor alone or as a multichaperone complex. Taken together, a proposed model for negative regulation of HSF1 by chaperones (Figure 3) is to be conclusively demonstrated by using *in vivo* assays and *in vitro* reconstitution assays to fully reproduce the transitions of HSF1 structure and activity.

Recently, a novel conserved 76-amino-acid protein termed Heat Shock Factor Binding Protein 1 (HSBP1) was identified in a yeast two hybrid protein interaction assay using the hydrophobic repeats of the trimerization domain of the mouse HSF1 as a bait (Satyal *et al.*, 1998). HSBP1 is a nuclear protein that interacts *in vivo* with the active trimeric HSF1. Interestingly, during attenuation of HSF1 to inert monomer, HSBP1 associates with hsp70. HSBP1 was shown to reduce the DNA-binding activity of HSF1 when both are coexpressed in reticulocyte lysate, while overexpression of HSBP1 in mammalian cells represses endogenous HSF1 transactivation, as judged from transcriptional assays. The negative effect of HSBP1 on the transcriptional response of HSF1 was also seen in HSBP1 transgenic *Caenorhabditis*

*elegans* strains where HSBP1 blocked activation of the heat shock response from a heat shock promoter-reporter construct, providing a biological role for this protein. These results do not, however, provide conclusive evidence for a role of HSBP1 in the heat shock response. It is not clear at which level HSBP1 exerts its biological effect since the data do not exclude the possibility that HSBP1 is also associated with inactive, monomeric HSF1. In addition, more experiments should be performed to establish the biochemical parameters for HSF1 interaction with HSBP1 and to understand the nature of HSBP1 inhibitory effect on HSF1 DNA-binding ability. Apart from chaperones and HSBP1, there are no other known HSF1 interactors. HSF1 is regulated at multiple levels (DNA binding, oligomerization, transcriptional activity) and it is formally possible that other, as-yet-unknown components, such as a small ligand, or a metabolite, or covalent modification of the protein play a role at each level. Further progress may be obtained through genetic screens for new cellular regulators and systematic analysis of reagents that affect HSF1 function.

## Physiological functions of HSF

As mentioned above, yeast and *Drosophila* HSF and their functional equivalent in vertebrates, HSF1 is the major stress-inducible transactivator of the heat shock response. Genetic studies indicated a pleiotropic role for the single copy HSF gene in both *Saccharomyces cerevisiae* and *Drosophila*. The

HSF of the yeast *Saccharomyces cerevisiae* is required *in vivo* for the induction of hsp synthesis in response to heat stress (Smith and Yaffe, 1991). Interestingly, yeast HSF is also essential for cell growth at normal temperature (15-30°C), which may be related to the regulation of basal hsp gene expression (Sorger and Pelham, 1988). Sorger and Pelham (1988) provided genetic evidence that HSF in yeast is essential at normal growth temperatures. Others also reported that at least some copies of the major hsp genes, such as hsp70, are expressed in yeast cells under normal growth conditions (Werner-Washburne *et al.*, 1987; Sorger, 1991). More insights into the function of HSF came from the work of Jedlicka and colleagues who showed that although *Drosophila* HSF is indispensable for the heat shock response *in vivo*, it is not essential for general growth and viability. However, it is required under normal growth conditions for oogenesis and early larval development as well as for survival under extreme stress conditions (Jedlicka *et al.*, 1997). The requirement for HSF in oogenesis and early development does not appear to be related to the regulation of hsp gene expression and thus may involve the regulation of novel, non-heat shock genes. This is in agreement with the previously published studies, which indicated that the developmental expression of hsps in *Drosophila* is independent of HSEs (Xiao and Lis, 1989). In addition, since these two requirements for HSF function (oogenesis and early larval development) in *Drosophila* are genetically separable, these target genes may not be the same. Screens for genetic interactions and analysis of differential

gene expression in *Drosophila* should allow the identification of other components of the developmental HSF pathway and also provide clues to the potentially important role of HSF in vertebrate development.

In mammals, HSF1 is not essential under normal conditions of growth, but is required for inducible hsp gene expression *in vivo* and acquired thermotolerance (McMillan *et al.*, 1998). This function is consistent with similar requirements for the single-copy homologue in lower Eukaryotes. Thermotolerance is defined as the ability of an organism to withstand extreme heat stress and correlates well with the induced level of hsp expression in yeast and *Drosophila* (Sewell *et al.*, 1995; Velasquez and Lindquist, 1984). *In vitro* studies using mouse embryonic fibroblasts (MEFs) have demonstrated that a HSF1 null mutation completely abrogated the capacity of HSF1  $-/-$  cells to acquire thermotolerance and thereby significantly increased their susceptibility to, and reduced their protection against, heat-induced apoptosis. However, unlike heat-stress induced expression, constitutive expression of hsps was not affected (McMillan *et al.*, 1998). In addition, other members of the HSF family did not compensate for loss of heat shock responsiveness. These findings provided the first direct evidence that constitutively expressed and inducible hsp family members exhibit distinguishable functional roles in cell physiology. More detailed analyses of the HSF1  $-/-$  deficient mice demonstrated that these animals exhibit multiple phenotypes, including: defects of the chorioallantoic

placenta and prenatal lethality; growth retardation; female infertility; and exaggerated tumor necrosis factor alpha (TNF- $\alpha$ ) production resulting in increased mortality after endotoxemic and inflammatory challenge (Xiao *et al.*, 1999). The unexpected finding that HSF1 is required for development of chorioallantoic placenta and postnatal growth needs to be fully elucidated, but based on immunohistochemical studies, Xiao and colleagues hypothesized that it may not be related to the regulation of hsp gene expression. Therefore, it is possible that HSF1, like *Drosophila* HSF, can regulate non-hsp genes in placental mammals. Previous studies have indicated that vertebrate HSFs play a role in spontaneous hsp gene expression during embryogenesis, development and postnatal growth (Tanguay *et al.*, 1993; Christians *et al.*, 1997). However, more thorough analyses is needed to determine the role of HSF1 in postimplantation development through identification of HSF1 target genes and signaling pathways regulated by the factor.

A direct role for HSF1 deficiency in pathological states in mice was proposed based on increased mortality and exaggerated TNF- $\alpha$  production during endotoxemia (Xiao *et al.*, 1999). Previous data demonstrated that pre-treatment with either heat shock or chemicals which induce hsp expression improved the survival of animals treated with endotoxin (Hotchkiss *et al.*, 1993). A role for HSF1 in the regulation of cytokine gene expression has been proposed previously (Cahill *et al.*, 1996). These researchers found that HSF1 acts as a transcriptional repressor of prointerleukine 1 $\beta$

gene expression in human monocytes treated with endotoxin. This effect was strictly dependent on an HSE in the prointerleukin 1 $\beta$  promoter. Thus, increased production of pro-inflammatory cytokine TNF- $\alpha$  in HSF-/- animals provided direct evidence for an *in vivo* role of HSF1 in transcriptional repression. This suggests an additional cytoprotective role for HSF1 *in vivo* during pathological events.

In conclusion, gene targeting experiments provided evidence for multiple functional roles for HSF1 *in vivo*. Some of these roles appear to be unrelated to its characteristic function, as a stress-responsive transcriptional activator and should stimulate further study of the function and regulation of HSF1 in cell homeostasis.

## The cell biology of HSF1 activation

At the cellular level, HSF distribution has been studied by either a biochemical approach (using cell fractionation and subsequent analysis of nuclear and cytoplasmic fraction in a gel mobility shift or Western blot assay), or by indirect immunofluorescence on fixed cells. These experiments have provided consistent results with respect to the subcellular distribution of the active trimeric *Drosophila* HSF or mammalian HSF1, which was always found to be nuclear upon heat shock (Larson *et al.*, 1988; Mosser *et al.*, 1988; Zimarino *et al.*, 1990; Westwood *et al.*, 1991; Sarge *et al.*, 1993; Baler *et al.*, 1993; Sistonen *et al.*, 1994; Zuo *et al.*, 1995; Mercier *et al.*, 1998). Earlier studies have revealed that upon exposure of



HeLa cells to heat shock, HSF1 forms brightly staining nuclear foci or granules (Sarge *et al.*, 1993). These structures, whose appearance correlates with HSF1 activation, are characterized in more detail in subsequent studies (Cotto *et al.*, 1997; Jolly *et al.*, 1997, 1999), in which it was shown that they can be induced by stresses other than heat shock, such as amino acid analogues or heavy metals. The results of these studies also showed that granules can be detected in living stressed cells using green fluorescent protein (GFP) tagged HSF1. Furthermore, the kinetics of their formation parallels the transient induction of heat shock gene transcription. During the recovery from heat stress, granules are no longer detected but HSF1 rapidly localizes to the same sites during a subsequent reexposure to heat shock. Fluorescence recovery after photobleaching (FRAP) experiments revealed that the HSF1 trimers are mobile within the dimensions of the granules, yet HSF1 can not be extracted readily from these structures by detergent permeabilization and salt extraction of heat shocked cells. One prominent feature of heat shock granules is that they can be detected only in primate cells, either primary or transformed. Secondly, immunostaining experiments revealed that HSF1 stress granules do not coincide with other previously described nuclear compartments such as nucleoli, coiled bodies, kinetochores, promyelocytic leukaemia (PML) bodies or the speckles enriched in splicing factors (Cotto *et al.*, 1997). Fluorescent *in situ* hybridization experiments gave contradictory results with respect to colocalization of granules with transcription

sites for hsp70 and hsp90 genes. However, the number of HSF1 granules correlates with ploidy of cells, which suggests the existence of specific chromosomal targets (Jolly *et al.*, 1997; He *et al.*, 1998). The functional significance of HSF1 stress granules is not clear. It was proposed that they define a compartment for the regulation of HSF1 activity which may function at multiple levels, such as inducible phosphorylation, association with chaperones or HSBP1 and changes in oligomeric state (Jolly *et al.*, 1999). In this way, the granules may be sites for storage of active HSF1 from which the factor is recruited to the loci of active transcription. Alternatively, HSF1 granules may represent sites where the factor is engaged in an activity distinct from transcription, playing a structural role in the chromatin changes during heat stress. This view was supported by experiments based on bromodeoxyuridine (BrUTP) incorporation, in which, only a very low level of incorporation into the granules was detected (Jolly *et al.*, 1999). In addition, the granules form independently of active transcription since they can be detected in heat shocked mitotic cells in which there is no heat shock transcriptional response, despite the activation of HSF1 DNA-binding activity (Milarsky *et al.*, 1986; Mosser *et al.*, 1993; Martinez-Balbàs *et al.*, 1995). Interestingly, other heat shock factors (HSF2, 3 and 4) have also been visualized as nuclear foci upon overexpression (Sheldon and Kingston, 1993; Nakai *et al.*, 1995, 1997), without correlation with gene activation or heat shock response.

In contrast to the situation observed with the active HSF, the subcellular localization of the inactive HSF is still an open subject. Biochemical fractionation showed cytoplasmic localization of HSF in *Drosophila* and HSF1 in mammalian cells under physiological conditions (Larson *et al.*, 1988; Mosser *et al.*, 1990; Zimarino *et al.*, 1990; Westwood *et al.*, 1991; Sarge *et al.*, 1993; Baler *et al.*, 1993). This led to the proposal that heat shock induced nuclear translocation of HSF and that this event represents a step in the activation of HSF. By contrast, immunofluorescence staining of unstressed fixed cells showed either cytoplasmic (Zandi *et al.*, 1997), or nuclear localization of *Drosophila* HSF (Westwood *et al.*, 1991; Orosz *et al.*, 1996). Its mammalian counterpart HSF1 was detected either in both the nucleus and cytoplasm (Sarge *et al.*, 1993), or as a predominantly nuclear protein (Rabindran *et al.*, 1991; Martinez-Balbàs *et al.*, 1995; Cotto *et al.*, 1997; Jolly *et al.*, 1997, 1999; Mercier *et al.*, 1998). The possible explanations for these discrepancies are discussed in detail below. Briefly, some of this difference can be explained by the fact that upon cell homogenization many nuclear proteins were found to "leak out" into the cytosolic fraction (Paine *et al.*, 1983), and this "leakage" is probably more acute with the inactive than with the active form of HSF1. On the other hand, the discrepancies seen in immunofluorescence analysis can be traced to the difference in the antibodies used, which probably reflect their specificity for HSF subpopulations in the cell (Morimoto *et al.*, 1994).

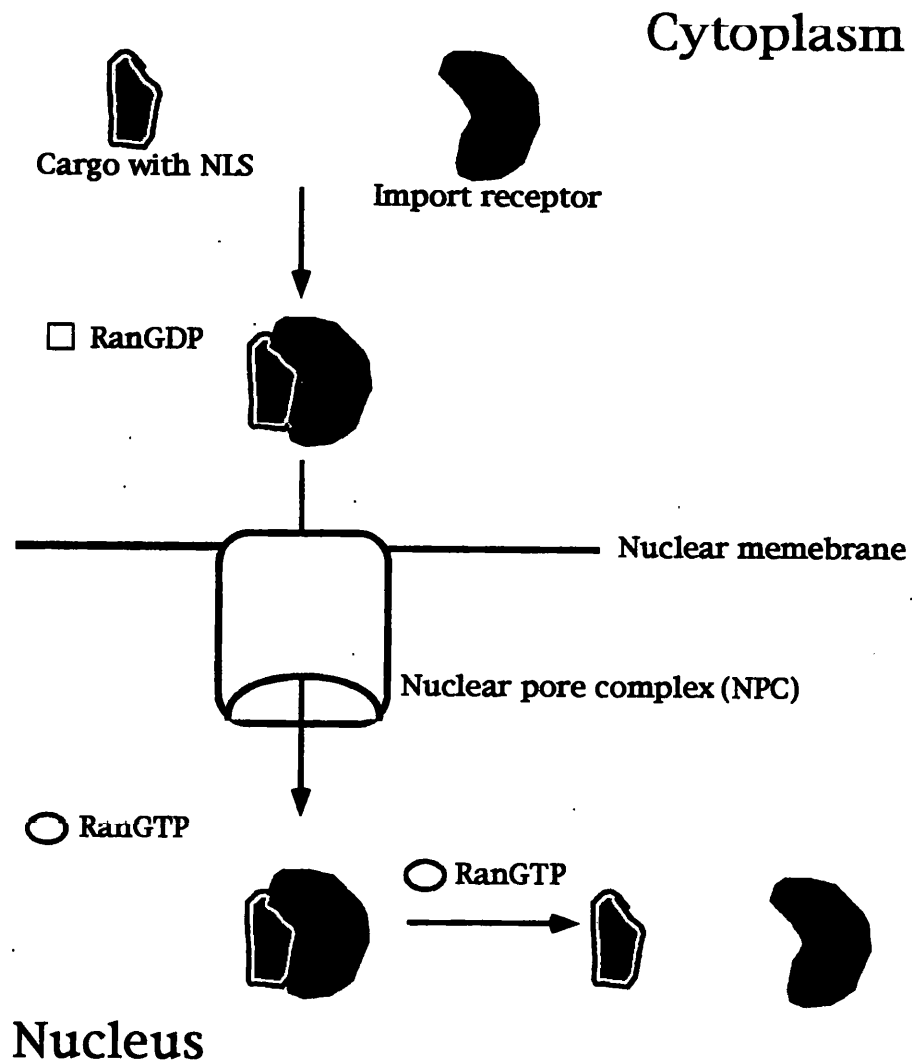
Finally, a report of Wang and Lindquist (1998), results obtained with the HSFA2 from tomato (Heerklotz *et al.*, 2000), as well as data presented in this work suggest that the intracellular localization of HSF is dynamic and that HSF has the ability for nucleocytoplasmic shuttling. Wang and Lindquist showed that heat shock-specific transcription in *Drosophila* early embryos is controlled by the developmentally programmed relocalization of HSF from the cytoplasm to the nuclei. Heerklotz and colleagues reported that steady state cytoplasmic localization of tomato HSFA2 is due to the nuclear export mediated by the leucine-rich nuclear export signal (Heerklotz *et al.*, 2000). The data shown here describe continuous nucleocytoplasmic shuttling of an inactive mammalian HSF1. This movement is reversibly discontinued during heat stress and resumes during the recovery from stress conditions. Taken together, these data suggest that nucleocytoplasmic shuttling of HSF1 may represent a novel mechanism for regulating the activity of the factor and thus may have implications for the regulation of the heat shock response (see Discussion).

## Nucleocytoplasmic transport

It is becoming evident that many biological processes are controlled by regulating the movement of macromolecules into and out of the nucleus. This exchange occurs through nuclear pore complexes (NPCs) (Nigg, 1997; Doye and Hurt, 1997; Ohno *et al.*, 1998), large multisubunit structures that

span both membranes of the nuclear envelope. NPCs form aqueous channels that allow passive diffusion of small molecules (up to 9 nm in diameter), while large molecules and complexes (up to 25 nm) are transported by an active (energy consuming) mechanism.

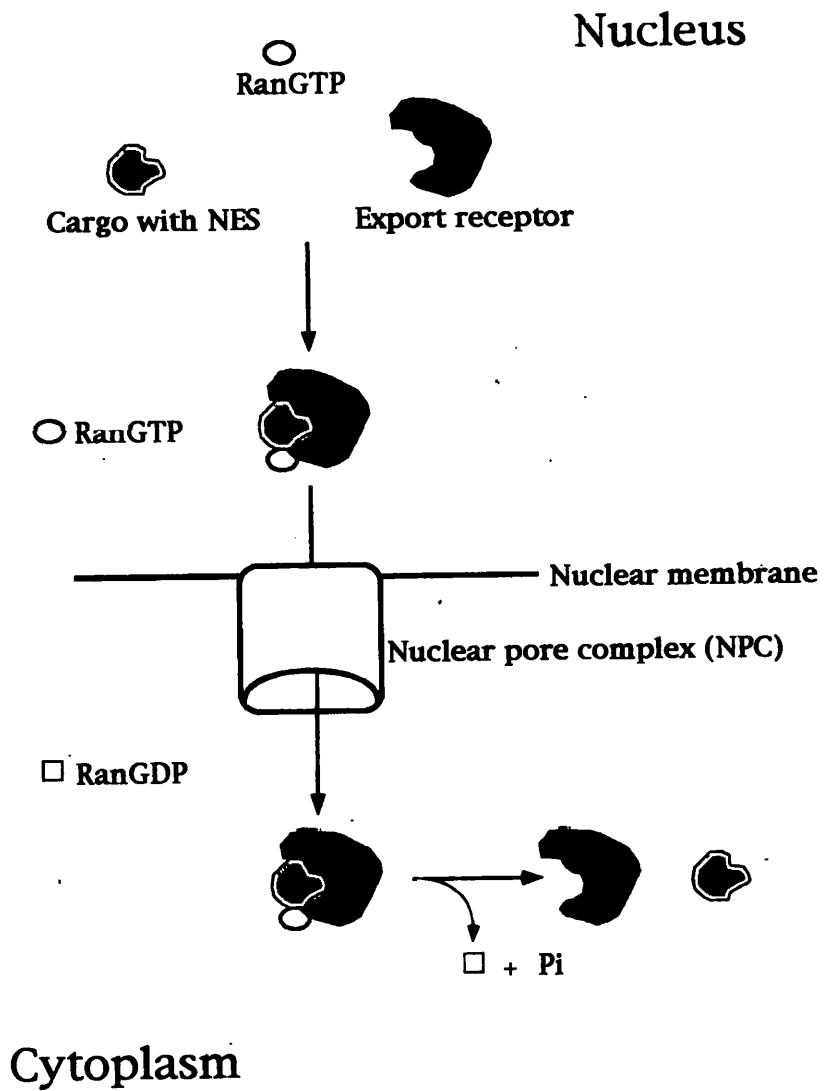
Signal-mediated nuclear import is a multistep process that requires energy, physiological temperature, a nuclear localization signal (NLS) on the substrate and soluble transport machinery. The "classical" nuclear localization signal is characterized by one or more clusters of basic amino acids first identified in the large T antigen of simian virus 40 (SV40) (Kalderon *et al.*, 1984) and nucleoplasmin (Dingwall *et al.*, 1982). A schematic diagram of the "classical" nuclear import pathway is shown in Figure 4. A protein carrying NLS is bound by a cytoplasmic receptor that consist of importins  $\alpha$  and  $\beta$  (Gorlich and Mattaj, 1996). There is a growing number of importin  $\alpha$  and importin  $\beta$  family members in mammalian cells that play a distinct role in nuclear targeting. Importin  $\alpha$  family members serve as adapters that bridge NLS-containing cargoes and importin  $\beta$ . Importin  $\beta$  targets the complex to the NPC; the complex is than translocated through the NPC into the nucleus where it is dissociated by the binding of Ran-GTP to importin  $\beta$ . Importantly, docking of the cargo-receptor complex at the cytoplasmic face of the NPC is energy-independent and can occur at low temperature (4°C), whereas translocation through the NPC depends on energy and temperature.



**Figure 4.** Schematic view of a "classical" nuclear protein import cycle. The NLS-bearing cargo forms a complex with the import receptor in the cytoplasm which first docks to the cytoplasmic periphery of the NPC, and is subsequently translocated to the nuclear side of the NPC. Import requires cytoplasmic Ran to be in its GDP-bound form. The translocation into the nucleus is terminated when protein cargo dissociates from the import receptor by direct binding of nuclear Ran-GTP to the import receptor.

Protein export shares many similarities with protein import. It also requires energy, physiological temperature, a nuclear export signal (NES) on the substrate and soluble transport factors. The best characterized pathway uses a leucine-rich nuclear export signal (NES), first identified in HIV REV protein and in protein kinase A inhibitor (PKI) (Fischer *et al.*, 1995; Wen *et al.*, 1995). In the nucleus, an NES-containing protein is bound by a soluble export receptor, known as CRM1 (Exportin1). Unlike importins, this binding requires the presence of Ran-GTP. The trimeric complex of NES-containing cargo, Exportin1 and Ran-GTP is then translocated to the cytoplasm, where it dissociates upon Ran-GTP hydrolysis (Figure 5). Like in the case of protein import, translocation of the trimeric complex through the NPC is an energy- and temperature-dependent step.

Recent data suggest that the Ran-GTPase cycle plays an essential role in dictating directionality of transport across the nuclear envelope (Gorlich *et al.*, 1996; Izauralde *et al.*, 1997). The cytoplasmic localization of Ran-GAP1, a principal Ran-GTPase activating protein (Hopper *et al.*, 1990; Matunis *et al.*, 1996) and the nuclear localization of a major Ran-GTP exchange factor, chromatin bound RCC1, (Ohtsubo *et al.*, 1989; Bischoff and Ponstingl, 1991) predict a high concentration of Ran-GTP in the nucleus and a low concentration of Ran-GTP in the cytoplasm (Gorlich *et al.*, 1997). This asymmetric distribution of Ran-GTP is further facilitated by two cytoplasmic proteins, Ran-binding proteins 1 and 2 (RanBP1, RanBP2) (Bischoff *et al.*, 1995). It is thought



**Figure 5.** Schematic view of Exportin 1-mediated nuclear protein export. The formation of the export receptor-cargo complex is supported by high level of RanGTP in the nucleus. Once in the cytoplasm, RanGTP is converted to its GDP-bound form and the export-cargo complex disassembles.



that this gradient determines directionality of transport by regulating the stability of transport receptor-cargo complexes; importin-cargo complexes form in the cytoplasm and dissociate in the nucleus, whereas exportin-cargo-Ran-GTP complexes form in the nucleus and dissociate in the cytoplasm. The RanGTPase cycle has also been proposed to mediate protein translocation through the NPC by GTP hydrolysis. However, there is evidence suggesting that certain substrates can be transported into and out of the nucleus in the absence of Ran-mediated GTP hydrolysis (Nakielnny and Dreyfuss, 1998; Ribbeck *et al.*, 1999).

In recent years it has become evident that multiple pathways exist for protein import and export. Apart from the "classical" NLS and leucine-rich NES, other signals driving nuclear import or export of proteins, have been described and characterized to varying extents (Kambach and Mattaj, 1992; Michael *et al.*, 1995, 1997; Truant *et al.*, 1998). In some cases, importin  $\beta$ -related import and export receptors mediating the transport through these signals have been identified (Siomi *et al.*, 1997). These studies have revealed complex regulation of protein trafficking in Eukaryotic cells. For example, some import pathways do not utilize importin  $\alpha$  adapter, but rather import cargo bound directly to one of the importin  $\beta$  family members (Palmeri and Malim, 1999). Several import receptors have been shown to recognize and import more than one kind of NLS (Jakel and Görlich, 1998). Furthermore, it appears that in some cases translocation of either a receptor or receptor-

cargo complex through the NPC is independent of Ran and energy (Nakielnny and Dreyfuss, 1998; Ribbeck *et al.*, 1999).

## Nucleocytoplasmic shuttling

Unlike proteins that reside constitutively in the nucleus or in the cytoplasm, some proteins move continuously back and forth. This behaviour is termed nucleocytoplasmic shuttling and has been observed for nucleolar proteins, RNA-binding proteins, viral regulatory proteins, transcription factors and nuclear import/export receptors. In many cases, shuttling is mediated by combined actions of the NLS and the NES in a protein, such as, for example, shuttling of p53 (Middeler *et al.*, 1997; Stommel *et al.*, 1999), NF-AT (Zhu and McKeon, 1999), c-Abl tyrosine kinase (Taagepera *et al.*, 1998) or viral regulatory proteins Rev (Meyer and Malim, 1994), E1B and E4 (Dobbelstain *et al.*, 1997). For other shuttling proteins, signals have not yet been characterized. It was proposed that the NLS of the progesterone receptor mediates both nuclear import and export (Guichon-Mantel *et al.*, 1994), but this observation was not further characterized. An emerging class of transport signals known as nucleocytoplasmic shuttling (NS) signals can also control shuttling. NS signals, unlike NLS and NES, can direct both nuclear import and nuclear export. The best characterized NS signal is the M9 domain, a 38 amino acid sequence that controls trafficking of hnRNPA1 (Michael *et al.*, 1995). All NS-containing proteins identified so far are mRNA binding

proteins (Michael *et al.*, 1997; Fan *et al.*, 1998; Tang *et al.*, 1999).

With respect to the steady state subcellular localization, shuttling proteins are found to be either predominantly nuclear or cytoplasmic or distributed in both compartments. The steady state distribution of shuttling proteins probably reflects the rate at which these proteins are imported into and exported from the nucleus. Therefore, for the shuttling proteins that are predominantly nuclear at steady state (progesterone receptor, p53, hnRNPA1) the lack of cytoplasmic staining in immunofluorescence experiments can be explained by the speed of bidirectional movement which causes them to be present in the cytoplasm only transiently.

Nucleocytoplasmic shuttling has been studied by using different transport assays such as formation of transient interspecies heterokaryons or nuclear microinjection in *Xenopus* oocytes. The transient interspecies heterokaryon assay has proven to be particularly useful because it allows easy identification of shuttling proteins (either endogenous or overexpressed) by immunofluorescence. In addition, it is possible to delineate an NES in such proteins, even if it is coincident with the NLS. On the other hand, *Xenopus* oocyte nuclear injection requires the use of recombinant proteins, does not allow easy identification of shuttling proteins, but allows quantitative analysis of both import and export pathways and their characterization through competition experiments (Nakielnny and Dreyfuss, 1997). Initial studies combining these two assays have demonstrated that major

nucleolar proteins, nucleolin, Nopp140 and NO38 are shuttling proteins (Borer *et al.*, 1989; Schmidt-Zachman *et al.*, 1993). These proteins showed very slow kinetics (24 hours) of internuclear transfer in heterokaryons or nuclear export upon microinjection. The signals, mechanism and role of this pathway are still unknown, but based on these findings it was proposed that shuttling ability of nuclear proteins is primarily determined by intranuclear interactions, rather than positively acting export signals. However, subsequent experiments showed that for most other shuttling proteins this was not the case. Rather, nucleocytoplasmic shuttling is a regulated process that was postulated to have a role in cellular functions. This was inferred from the fast shuttling (export) kinetics in the heterokaryon assay, or upon microinjection in *Xenopus* oocyte nucleus. For example, two hsp70-related proteins of *Xenopus* and the rat heat shock cognate (hsc70) were exported within 2 hours of microinjection (Mandell and Feldherr, 1990). Overexpressed p53 apparently equilibrates between nuclei of heterokaryons 3 hours post-fusion (Stommel *et al.*, 1999). Glucocorticoid receptor shows similar export kinetics (4 hours in heterokaryon assay), whereas many RNA-binding proteins (hnRNPA1) and viral regulatory proteins (HIV Rev) showed a very fast export rate in this assay (1 hour or 45 min, respectively). Identification of NLS and NES signals further helped to characterize shuttling proteins. It turned out that many of them have leucine-rich NES (p53, HIV Rev, actin, MAPKK) which is recognized by the Crm1 (Exportin 1) nuclear

export receptor. Furthermore, the discovery of Leptomycin B (LMB), which specifically inhibits Exportin 1 helped to identify more proteins containing leucine-rich NES. Many of these proteins (actin, interferon regulatory factor-3, MEK1) are predominantly cytoplasmic at steady state. Such a distribution is probably the result of a high export rate since LMB treatment causes nuclear accumulation. Therefore, it is assumed that these proteins shuttle continuously between the nucleus and the cytoplasm *in vivo*. Components of the nucleocytoplasmic transport machinery; importins and exportins, are themselves shuttling proteins. After delivery of a protein cargo they are transferred back to the original compartment for a new round of cargo loading and transport. For example, upon dissociation from a protein cargo in the nucleus, importin  $\alpha$  is transported back to the cytoplasm by the action of the specific export receptor CAS (Kutay *et al.*, 1997). However, the importin  $\alpha$  NES is not yet identified.

## Functional implications of shuttling

As mentioned above, nucleocytoplasmic shuttling is a function common to many proteins belonging to different classes, such as mRNA-binding proteins, splicing factors, viral regulatory proteins, transcription factors, tyrosine kinase receptors and structural proteins. What is the role of their continuous movement? For shuttling mRNA-binding proteins, it was suggested that they play a role in mRNA export from the nucleus, since electron microscopy experiments showed

that mRNA export occurs in the context of ribonucleoprotein (RNP) particles (Mehlin *et al.*, 1992, 1995). Similarly, shuttling of SR proteins could facilitate mRNA transport through the NPC or have cytoplasmic functions, such as on mRNA stability, localization or translational regulation (Càceres *et al.*, 1997). Considering their abundance and fast shuttling kinetics, both hnRNPs and SR proteins can also act as carriers of proteins to both compartments. Another possibility is that transient passage through the cytoplasm may allow their nuclear activities to be regulated by some cytoplasmic components such as enzymes, as proposed previously for shuttling nucleolar proteins (Borer *et al.*, 1989). Viral regulatory proteins such as HIV Rev play an essential role in the nuclear export of intron-containing viral transcripts (Felber *et al.*, 1989; Malim *et al.*, 1989). A similar role was also proposed for shuttling of adenoviral oncoproteins E1B and E4 (Dobbelstain *et al.*, 1997). On the other hand, nucleocytoplasmic shuttling of cyclin B1 was proposed to be an additional mechanism for coordinating nuclear and cytoplasmic aspects of entry into mitosis (Yang *et al.*, 1998; Hagting *et al.*, 1998). In addition, a possible role of cyclin B1 nuclear export has been proposed for the DNA damage-induced G<sub>2</sub> checkpoint (Toyoshima *et al.*, 1998). NES mediated nuclear export of actin may be utilized to ensure cytoplasmic localization, i.e. nuclear exclusion under normal conditions, since the presence of actin in the nucleus can be harmful to the cell (Wada *et al.*, 1998). However, the existence of a nuclear pool of actin has also been discussed (Rando *et*

*al.*, 2000) and it is possible that shuttling of actin has an additional role in cell physiology.

## **The role of nucleocytoplasmic transport and shuttling in transcriptional regulation**

Nuclear import and export may represent important regulatory checkpoints in the control of gene expression by transcription factors. A number of studies indicate that this regulation is exerted by transcription factors at the level of the transport substrate. Some transcription factors are anchored either in the cytoplasm or in the nucleus through binding to other structures or partners. For example, sterol regulatory element binding protein (SREBP) is inserted in the membrane of the endoplasmic reticulum. Upon sterol depletion, SREBP is proteolytically cleaved and a portion of SREBP containing the DNA-binding domain, basic NLS and a transactivation region is translocated to the nucleus where it activates transcription of target genes (Brown *et al.*, 1997). Cytoplasmic anchoring was also described for a temperature-sensitive mutant of p53 Val135, which accumulates in the cytoplasm at restrictive temperatures due to its binding to the cytoskeleton: at permissive temperatures, this protein accumulates in the nucleus (Klotzsche *et al.*, 1998). In a similar manner, association with its specific chromatin targets prevents nuclear export of the glucocorticoid receptor in the presence of hormone. Upon hormone withdrawal, there is a

rapid release of the receptor from chromatin followed by nuclear export back to the cytoplasm (Yang *et al.*, 1997).

In other cases, regulation of nucleocytoplasmic localization is achieved through masking of NLS/NES, either as a consequence of conformational change or by association with an interaction partner. Two examples of such regulation are the yeast transcription factor Pho4 and mammalian transcription factor NF-AT. The subcellular distribution of these proteins is modulated through post-translational modifications i.e. phosphorylation and dephosphorylation. In the absence of phosphate in the growth medium, Pho4 is underphosphorylated and directly interacts with its nuclear import receptor. Once in the nucleus, Pho4 activates transcription of phosphate-responsive genes. In the presence of phosphate in the growth medium, nuclear Pho4 becomes phosphorylated, interacts with its nuclear export receptor Msn5 and is exported to the cytoplasm where phosphorylation inhibits its interaction with the import receptor (Kaffman *et al.*, 1998). A similar mechanism of dephosphorylation-dependent NLS unmasking coupled to phosphorylation-dependent NES unmasking also regulates the nucleocytoplasmic distribution of NF-AT. In resting T cells, NF-AT is cytoplasmic, but upon TCR and CDC28 coreceptor activation, increased intracellular calcium triggers NF-AT dephosphorylation, NLS unmasking and subsequent nuclear import. Upon return of calcium to resting levels, nuclear NF-AT is phosphorylated allowing NES to be exposed and NF-AT is exported back to the cytoplasm by the Crm1 nuclear export



receptor (Crabtree, 1999). In contrast, oxidation of Yap1p, a yeast AP-1-like transcription factor inhibits its binding to Crm1 (Yan *et al.*, 1998), resulting in nuclear accumulation. Another example of regulated protein export in the fission yeast is provided by the transcription factor Pap1. Under conditions of oxidative stress its interaction with Crm1 is disrupted and Pap1 is transported to the nucleus; this translocation is dependent on stress-activated MAP kinase Sty1 (Toone *et al.*, 1998).

NLS masking can also be achieved through the association of a transcription factor with an inhibitor. The best characterized example is the NF- $\kappa$ B transcription factor complexed with inhibitory molecules, I $\kappa$ Bs, that directly mask NLS of NF- $\kappa$ B. The cytokine and stress-induced stimulation leads to the I $\kappa$ B phosphorylation, ubiquitination and degradation. NF- $\kappa$ B interacts with nuclear import receptor and enters the nucleus (Baeuerle and Baltimore, 1996). Similarly, hormone binding to the glucocorticoid receptor in the cytoplasm triggers dissociation of inhibitory chaperone hsp90 and NLS unmasking (Pratt, 1992).

NLS unmasking and nuclear translocation can be triggered also by interaction with an activator protein, as in the case of mammalian MAP kinase ERK1 that translocates into the nucleus upon phosphorylation and homodimerization (Khokhlatchev *et al.*, 1998). The leucine-rich NES of PKI, a specific inhibitor of the catalytic subunit of cAMP-dependent protein kinase, is exposed only when PKI binds to the

catalytic subunit of the kinase in the nucleus promoting its nuclear export (Wen *et al.*, 1995).

Finally, some transcription regulatory proteins not containing any NLS or NES interact with NLS- or NES-bearing partners. This transport mechanism is called "piggy-back". For example, pancreatic transcription factor 1 (PTF-1), which exists in the cytoplasm as a dimer, is imported to the nucleus through an interaction with the NLS containing protein p75 (Sommer *et al.*, 1991).

Taken together, these examples illustrate that regulated nucleocytoplasmic transport of transcriptional regulatory proteins is a common mechanism in the control of gene expression. Apart from playing a role in controlling localization of transcription factors, nucleocytoplasmic shuttling could also have other implications for their function. For nuclear transcription factors such as progesterone receptor, p53 and HSF1, the transit through the cytoplasm provides the opportunity for continuous sampling of the cytoplasmic environment. Since these transcription factors appear to shuttle continuously, they can receive and integrate inputs from both compartments. This could be achieved through an interaction with compartment-specific regulators such as enzymes, ligands or metabolites. For example, compartment-specific post-translational modification may play a role in modulation of their activity. In the case of p53, it was proposed that nuclear export, mediated by hdm2, targets p53 for degradation in the cytoplasm (Roth *et al.*,

1998). Alternatively, shuttling transcription factors may have an unknown function in the cytoplasm.

In conclusion, further characterization of transport receptors and pathways utilized by transcription factors, as well as development of inhibitors of their activity, is likely to uncover the precise role for shuttling in transcription factors function.

## MATERIALS AND METHODS

### Growth and manipulations of *E.coli*

Procedures were as described by Sambrook *et al.*, 1982. The following strains were used: sNM522 ( $F'lac^qD(lacZ)M15proA^+B^+/SupEthi\Delta(lac-proAB)\Delta(hsdMS-mcrB)5(rk^-mk^-McrBC)$ ), HB101( $F^-\Delta(gptPROA)62leusupE44$   $ara14galK2lacY1\Delta(mcrC-mrr)rpsL20(Str^r)xyl5mtl-1recA13$  and MC1061/P3 ( $F^-araD139 \Delta(ara-leu)7696 galE15 galK16 \Delta(lac)X74 rpsL(Str^r) hsdR2(rk^-mk^-) mcrAmcrB1$  (Invitrogen). Strains were stored as 50% glycerol stocks at  $-80^\circ\text{C}$ .

### Preparation of competent bacteria and transformation

10 ml of Luria Bertani (LB) medium (1% bactotryptone; 0.5% yeast extract; 0.5% NaCl; all purchased from BDH) were inoculated with a single colony from a freshly streaked LB agar plate and incubated overnight (O/N) at  $37^\circ\text{C}$  with shaking at 225 rpm. NM522 or MC1061/P3 required medium containing Tetracycline or Kanamycin, respectively. On the next day, 5 ml were used to inoculate 500 ml of medium in a 2L flask. Cultures were grown at  $37^\circ\text{C}$  until  $A_{436} = 0.5-0.6$  (approximately 3-4 hours). After immersion in ice-water bath ( $0^\circ\text{C}$ ) for 30 min, cells were pelleted at 6000 rpm for 20 min at  $4^\circ\text{C}$ . The pellet was resuspended in 200 ml ice-cold  $\text{CaCl}_2$  solution (100 mM) and the suspension was incubated in ice for 30 min and then centrifuged at 6000 rpm for 15 min at  $4^\circ\text{C}$ . This step was repeated twice except that pellet resuspension was in 45 ml and then 5 ml of ice-cold  $\text{CaCl}_2$ . After addition of 1.2 ml pre-

chilled 50% glycerol, suspension was mixed gently by pipetting up and down with a blue tip and aliquoted (100  $\mu$ l). Aliquots were quickly frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Cells prepared this way are competent for at least 3 months.

For transformation, an aliquot of competent cells was thawed on ice for 5 min, mixed with plasmid DNA or an aliquot of ligation mixture (usually 1/3 of ligation mixture, i.e. 10  $\mu$ l), gently flicked and incubated at room temperature for 15 min. Cells were plated on LB-agar plates with appropriate antibiotic selection and incubated O/N at  $37^{\circ}\text{C}$ . The antibiotics were used at the following concentrations: Ampicillin (Sigma) 150  $\mu\text{g/ml}$ , Tetracycline 20  $\mu\text{g/ml}$  and Kanmycin (Boehringer) 40  $\mu\text{g/ml}$ . MC1061P3 strain was plated on plates containing 30  $\mu\text{g/ml}$  Ampicillin and 7.5  $\mu\text{g/ml}$  Tetracyclin (Boehringer). Antibiotics stock solutions were 100 mg/ml Ampicillin, 10 mg/ml Kanamycin and 10 mg/ml Tetracyclin.

## DNA MANIPULATIONS

General manipulations with DNA were all done according to Sambrook *et al.*, 1982, with some modifications.

### Enzymatic manipulations of DNA

DNA *restrictions* with commercial buffers and enzymes (New England Biolabs, Fermentas, Boehringer Mannheim) were according to manufacturer's instructions. Usually, 5-10 units (U) of enzyme were used to digest 1  $\mu\text{g}$  of DNA for 60-90 min at appropriate temperature. Reactions were stopped by 6X loading buffer (20% (w/v) sucrose; 10 mM

Tris-Hcl pH8,0; 5 mM EDTA pH8,0; 0.1% bromphenol blue). When needed, *dephosphorylation* reactions were carried directly as follows. After heat inactivation of restriction endonuclease (70°C for 20 min), 10 µl of dephosphorylation buffer (Boehringer) and 2 U of Calf Alkaline Phosphatase (Boehringer Mannheim) were added and the volume was adjusted to 100 µl with water. The DNA was dephosphorylated for 60 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of 5 mM. Phosphatase was inactivated for 20 min at 65°C after which the sample was extracted twice with equal volume of phenol/chlorophorm (1:1). DNA was precipitated by addition of 1/10 vol of 3M CH<sub>3</sub>COONa pH5.2 and 2.5 vol of absolute ethanol. After incubation at -80°C for 15 min or -20°C overnight, DNA was pelleted by centrifugation for 10 min in microfuge at 4°C. The pellet was washed with 70% ethanol, dried at 37°C and dissolved in an appropriate volume of buffer. *Ligation* reactions were with T4 DNA ligase (Boehringer) and commercial buffer, according to manufacturer's instructions. Typically, 1-5 ng of vector was mixed with 100-150 ng of "insert" and incubation was performed for 4 hours at 16°C or overnight at 4°C. 1/3 of ligation mixture (10-12 µl) was typically used for transformation of competent cells as described above.

Agarose gel electrophoresis was in 1X TAE buffer (40 mM Tris-acetate pH7.2; 1 mM EDTA) at ambient temperature under constant voltage of 10V/cm in Wide Mini Sub Cell Apparatus (Bio-Rad) and using Bio-Rad Electrophoreis Power Supply. Ethidium bromide (10 µg/ml) was added prior to pouring the gel. Gels were observed under UV-GEN T<sup>M</sup> Transiluminator (Bio-Rad) and photographed using video copy processor (Mitsubishi).

## PURIFICATION AND QUANTITATION OF DNA

### Purification of DNA through Agarose Gel

After restriction, PCR products or plasmid fragments were run through agarose gel of appropriate concentration, the band of interest was illuminated with UV lamp (Spectroline) and excised with a clean scalpel. The band was then cut into small pieces that were inserted into a blue tip with a filter. The tip was placed into 1.5 ml eppendorf tube and centrifuged in microfuge at room temperature for 20 min at 6000 rpm. The soluble content of the gel was recovered from the eppendorf tube, extracted twice with the equal volume of phenol/chloroform (1:1) and as described above.

### Purification of Plasmid DNA.

Purification of plasmids on JETSTAR *anion exchange column* (Genomed) was according to manufacturer's protocol involving alkaline lysis of bacteria. 100 ml O/N bacterial culture was harvested by centrifugation for 20 min at 6000 rpm at room temperature. Cells were resuspended in 10 ml of solution E1 (50 mM Tris-HCl pH8.0; 10 mM EDTA pH8.0; RNase 10 µg/ml) and lysed upon addition of 10 ml of solution E2 (200 mM NaOH; 1% SDS) followed by gentle mixing until the lysate appeared homogenous. After neutralization with 10 ml of cold solution E3 (3.1M CH<sub>3</sub>COOK pH5.5) the lysate was cleared by centrifugation for 30 min at 15000 rpm at room temperature. The cleared lysate was applied on the column pre-equilibrated in solution E4 (600 mM NaCl; 100 mM CH<sub>3</sub>COOK pH5.0; 0.15% TritonX-100). The

column was washed with 60 ml of solution E5 (800 mM NaCl; 100 mM CH<sub>3</sub>COOK pH 5.0) and eluted with 15 ml of solution E6 (1.25 M NaCl; 100 mM Tris-HCl pH8.5). The DNA was precipitated and then dissolved in 0.5 ml of water. The concentration was determined spectrophotometrically by reading OD<sub>260</sub> nm of 1:100 dilution of DNA in water and using conversion factor 1OD<sub>260</sub>=50 µg/ml.

For plasmid preparation by *Cesium Chloride Density Gradient Centrifugation*, typically a bacterial pellet from 0.5L culture was resuspended in 10 ml of cold TES buffer (50 mM Tris-HCl pH7.5; 40 mM EDTA; 25% (w/v) sucrose). To this suspension, 1 ml of lysozyme (Sigma) was added (from 10 mg/ml freshly prepared stock kept at 4°C) and suspension was incubated for 10 min on ice after which 3.7 ml of 250 mM EDTA pH8.0 were added and incubation on ice continued for additional 5 min. At this point, 14.5 ml of cold Triton solution (made by mixing 1 ml of 10% TritonX-100, 31.5 ml of 25 mM EDTA pH8.0, 5 ml of 1M Tris-HCl pH8.0 and 62.5 ml of water) was added and the mixture was incubated on ice for 10 min. Debris was removed by centrifugation at 18,000 rpm for 30 min. After addition of 1/10 vol of 5M NaCl and 0.8 vol of isopropanol, the supernatant was in ice for 30 min and then centrifuged at 9000 rpm for 30 min at 4°C. The pellet was air dried and resuspended in 10 ml of 1XTE buffer (10 mM Tris-HCl pH8.0; 1 mM EDTA pH 8.0). 10 gr of CsCl was then dissolved in this solution and 150 µl of ethidium bromide was added from the stock solution (10 mg/ml dissolved in water). After centrifugation for 5 min at 2000 rpm the transparent solution was transferred to 10 ml ultra crimp plastic tubes (Sorvall), avoiding the floating debris. Tubes were closed with plugs and sealed with heat. The plasmids were banded for 18 hours at 50,000 rpm at room temperature in Sorvall Ultracentrifuge. The band corresponding



to supercoiled plasmid DNA was collected in a 10 ml plastic syringe connected to a 18-gauge needle. Usually, between 1-3 ml were collected. Ethidium bromide was removed by repeated extractions with the equal volume of isoamyl alcohol in a syringe, inverting the syringe few times until water phase (lower) became colorless (usually, three extractions were sufficient). The sample was then transferred to a dialysis tube (Sigma) and dialyzed O/N at 4°C against 1X TE buffer (ratio 1:2000). The DNA was then transferred in 1.5 ml eppendorf tube and precipitated.

### **Small Scale Plasmid DNA Preparation ("mini prep")**

The isolation of plasmid DNA from 1.5 ml cultures (mini-prep) was done by alkaline lysis. The bacteria from overnight LB culture were pelleted in 1.5 ml eppendorf tube for 1 min in microfuge at 13.000 rpm. The pellet was resuspended in 150 µl of 50 mM Tris-HCl pH 8.0 and 10 mM EDTA). Cells were lysed by adding 150 µl of 200 mM NaOH and 1%SDS, inverting the tube several times, after which 150 µl of 3M CH<sub>3</sub>COOK pH5.2 was added. The tube was again inverted several times and incubated in ice for 10 min. After centrifugation in microfuge for 3 min, supernatant was extracted once with 450 µl of chloroform/isoamyl alcohol (1:1). The aqueous (upper) phase was recovered and DNA was precipitated. After centrifugation, the DNA pellet was dissolved in 50 µl of water and incubated at 65 °C for 20 min to inactivate contaminating nucleases. 5 µl were used for restriction reactions. RNaseA (Boehringer Mannheim) was added at 10 µg/ml final concentration.

## DNA constructs

### Polymerase Chain Reaction (PCR)

PCR based mutagenesis was used for codon substitutions as well as to generate termini suitable for in frame fusions to c-Myc epitope, Pyruvate Kinase, Nucleoplasmin core and Green Fluorescent protein. PCR was with *Vent* DNA Polymerase (New England Biolabs) in 0.5 ml thin wall tubes (Sorenson), using a MiniCycler<sup>TM</sup> (MJ Research). Primers were purchased from DNA Technology (Denmark) or Primm (Italy) and stored as 10  $\mu$ M stocks at -20°C. Reactions (generally 100  $\mu$ l) were in 1X Thermopol buffer, 1  $\mu$ M primers (usually 23-33 nucleotides long), dNTPs 200  $\mu$ M each (GIBCO-BRL) and 1 U of *Vent* DNA Polymerase (0.5  $\mu$ l). Reactions were assembled on ice and overlaid with 100  $\mu$ l of mineral oil (Sigma). The amount of template was usually 1-2 ng. Denaturation step was at 95°C, while extension step was at 72°C. Annealing temperature ( $T_a$ ) was calculated for each primer according to the following formula:  $T_a$  (°C) =  $T_m$  (°C) -5, where  $T_m$  is the melting temperature of a giving primer calculated as  $4 (\%G+C) + 2 (\%A+T)$ . In case annealing temperatures for given pair of primers were different, the lower value of  $T_a$  was chosen. Also, if the predicted  $T_a$  for a given pair of primers was high (> 70°C), due to high G+C content,  $T_a$  was determined empirically by increasing the annealing temperature in PCR reaction by 2°C steps starting from 52°C. This helped to eliminate appearance of non-specific PCR products. Usually, 28 cycles were performed as follows: step1/ denaturation, 1 min; step2/ annealing, 1 min; step3/ extension, 1 min. In case PCR products longer then 1kb were synthesized, the extension step was prolonged to 2 min. In all cases initial denaturation for 3 min at 95°C

was included. After completion of PCR reactions, the aqueous was transferred into 1.5 ml eppendorf tube, extracted with 100  $\mu$ l of phenol/chloroform (1:1) and precipitated. DNA was dissolved, cleaved with appropriate restriction nucleases and gel purified prior to ligation.

### **Codon substitutions by PCR.**

This procedure for PCR based mutagenesis was essentially done as described by Ausubel *et al.*, 1995, with some modifications. It involves the production of two PCR fragments with few overlapping bases (9-20) and their mutual priming in a subsequent PCR reaction. Briefly, oppositely oriented primers were designed with mutations of codons, as indicated below. The primers overlapped for at least 9 bases that included mutagenized residues. Each was paired with T7 (5'-TAATACGACTACTATAGGG-3') or SP6 (5'-TATTTAGGTGACACTATAG-3') primers in order to generate two PCR fragments from plasmid C12A that carries the HSF1 open reading frame. PCR products were gel purified, mixed (50 ng of each), denatured, renatured and subjected to an extension step prior to PCR with T7 and SP6 primers. After extraction and precipitation, the product was restricted with EcoRI, gel purified and ligated to pcDNA3 vector (Invitrogen) linearized with EcoR I. This procedure was used for non tagged HSF1 mutants, while the direct ligation of PCR products was carried out for the triple mutant M387K/L391A/L394A in the context of GFP-HSF1, as described below.

## Generation of Plasmid Tet17

The pUHD 10-3 plasmid (gift of M.Gossen) contains seven repeats of the *tet* operator linked to a cytomegalovirus minimal promoter (Pcmv-1) upstream from a polylinker containing multiple cloning sites and a SV40 polyadenylation signal (Resnitzky *et al.*, 1994). A 1.9 Kb *EcoR* I fragment, containing the mouse HSF1 cDNA and including 5' and 3' UTRs (Sarge *et al.*, 1991) was subcloned in the *EcoR*I site of pUHD10-3. Correct orientation of HSF1 insert in construct Tet17 was determined with *Apa*L I digestion.

## Green Fluorescent Protein-HSF1 fusion constructs (GFP-HSF1s)

For fusions to green fluorescent protein (GFP), the open reading frame (ORF) of wild type (wt) HSF1 (codons 1 to 503) or mutants (HSF1:Δ6-80, HSF1:Δ160-172 and HSF1:H179R) was in each case amplified by PCR using upstream primer S1, corresponding to residues 1-5, (5'-GAAGATCTATGGATCTGGCCGTG-3') and downstream primer S2, corresponding to residues 499-503, (5'-GAAGATCTGGAGACAGTGGGGTC-3'), both with *Bgl* II sites at 5' ends (underlined). After *Bgl* II restriction and gel purification, the fragment was subcloned into pEGFPC1 plasmid (Clontech) linearized with the same enzyme. Positive clones were in all cases identified by restriction with *Nhe* I/*Stu* I enzymes and verified by sequencing. Since the amplified ORFs did not contain a stop codon, the fusion resulted in addition of 26 codons from pEGFPC1 polylinker. The residues were the following: Arg, Ser, Arg, Ala, Gln, Ala, Ser, Asn, Ser, Ala,

Val, Asp, Gly, Thr, Ala, Gly, Pro, Gly, Ser, Thr, Gly, Ser, Thr, Gly, Ser and Arg.

For making GFP-HSF1:M387K/L391A/L394A, PCR based mutagenesis described above was used. S1 primer was combined with primer LZ4mut~~down~~, corresponding to residues 384-394, (5'-CGCGTTGTCCGCAGATCTGTCCTTGGCATCCAG-3') to amplify a fragment of 1 kb while, in parallel, S2 primer was combined with primer LZ4mut~~up~~, corresponding to residues 384-394 (5'-CTGGATGCCAAGGACTCTAGAGCGGACAACGCG-3'), to obtain a PCR fragment of 500bp. The codon substitutions, shown in bold, were the following: T at position 1299 was changed into A, replacing the codon for methionine with an alanine codon; C at position 1310 was changed into G and T at position 1311 was changed into C, replacing the codon for leucine with an alanine codon; similarly, C at position 1320 was changed into G and T at position 1321 was changed into C, replacing the leucine codon with an alanine codon and destroying a *Pst* I site. Two more mutations were introduced that created an *Xba* I site (underlined). C at position 1306 was replaced with T, changing the serine codon TCC into TCT; A at position 1308 was changed into G and G at position 1309 was changed into A, replacing the codon for asparagine with an arginine codon. Both PCR fragments were restricted with *Bgl* II and *Xba* I, and inserted in a three-fragment ligation into *Bgl* II linearized pEGFPC1. The presence of mutations was verified by sequencing.

## Generation of Myc-Tagged HSF1 Constructs

For creating mycHSF1, S1 primer and primer ORFdown, corresponding to residues 499-503 and carrying an *EcoR* I site at the 5'

end (underlined) (5'-CGGAAATTCGGAGACAGTGGGGTC -3') were used to PCR amplify HSF1 ORF. PCR product of 1.5 kb was restricted with *Bgl* II and *EcoR* I and gel purified. This fragment was then subcloned into pCDNA3 containing myc tagged Nucleoplasmin core-SV40 T antigen Nuclear Localization Signal (myc-NPc-TNLS) (Nakeilny *et al.*, 1995), from which the NPc piece was removed by *BamH* I/*EcoR* I digestion. The resulting plasmid, myc-HSF1-TNLS, was restricted with *EcoR* I and *Xho* I to remove TNLS, gel purified, filled-in with Klenow enzyme (Fermentas) and blunt-end self ligated. Sequencing with SP6 specific primer have shown that HSF1 open reading frame is followed by six additional codons from the pCDNA3 polylinker (Glu, Phe, Arg, Ala, Cys and Ile). This construct was used as a template to make myc-tagged HSF1 carboxy-terminal deletions by PCR, using T7 primer and the following downstream primers (all with *EcoR* I site at 5' end): P382 corresponding to residues 378-382, (5'-GAGAATTCCCTAATCCAGGTGATCACT-3'); P266 corresponding to residues 261-266, (5'-GAGAATTCCCTAGGAGATTATGGG TCC-3'); P235, corresponding to residues 231-235, (5'-GAGAATTCCCTAATGGACATGCTCCAG-3'); P228, corresponding to residues 224-228, (5'-GAGAATTCCCTACTGTCGACCATACTT-3'); P225, corresponding to residues 221-225, (5'-GAGAATTCCCTAATACTTGGGCAC AGA-3'); P198, corresponding to residues 194-198 (5'-GAGAATTCCCTACTGCACCAGTGAGAT-3'); P167, corresponding to residues 163-167 (5'-GAGAATTCCCTAGGCCACGTTACGTG-3'); P141, corresponding to residues 137-141 (5'-GAGAATTCCCTACAACAGCCGGGTGAC-3'); P120, corresponding to residues 116-120 (5'-GAGAATTCCCTAGGTCACTTTCCTCTT-3'). All downstream primers contained an in frame stop codon (shown underlined). The resulted PCR fragments were all restricted with *Hind* III and *EcoR* I and

after gel purification ligated to *Hind* III/*EcoR* I digested pCDNA3 (Clontech). All constructs were verified by sequencing.

## Generation of Non-Tagged HSF1 Mutants

Non-tagged mutants were made by PCR directed mutagenesis as described above. For triple amino acid substitution mutant HSF1:KRK206/8AAA, two PCR fragments were made from C12A. A fragment of 700 bp was generated using primers SP6 and NLS downmut, corresponding to residues 201-208 (5'-C**GCGGCCG**CCACCCCCAGGATCC GG-3'); a fragment of 1.2 kb was generated using primers T7 and NLSupmut corresponding to residues 206-214 (5'-**GCGGCCGCG**ATCCCTCTGATGTTGAGT-3'). The point mutations, shown in bold, were the following: A at positions 755 and 756 were changed into G and C respectively, which replaces the codon for lysine with an alanine codon; A at position 758 was changed into G, and G at position 759 was changed into C and A at position 760 was changed into C, which replaces the codon for arginine with an alanine codon; A at positions 761 and 762 was changed into G and C respectively, which replaces the lysine codon with an alanine codon. These codon substitutions created a *Not* I restriction site (underlined). The fragments were gel purified, mixed and subjected to a second PCR using SP6 and T7 primers and conditions described above. The final PCR product of 1.9 kb was digested with *EcoR* I and after gel purification subcloned in pCDNA3 linearized with *EcoR* I. The orientation of the insert was verified with *Not* I restriction and the presence of the mutation was verified by sequencing.

To create triple amino acid substitution mutant HSF1:KRK116/8AAA, two PCR fragments were made on C12A plasmid using the following set of primers: SP6 specific and 116/8downmut, corresponding to residues 116-123 (5'-**CGCGCCGC**CACTGGTCGCACAGG-3') which generated a fragment of 500 bp. In the other PCR reaction, a fragment of 1.4 kb was amplified using T7 specific and 116/8upmut primer, corresponding to residues 111-118 (5'-**GCGCGGCG**TGTGACCAGCGTGTCC-3'). The codon substitutions (shown in bold) were the following: A at positions 485 and 486 was changed into G and C respectively and G at position 487 was changed into C replacing the lysine codon with an alanine codon; A at position 488 was changed into C replacing the arginine codon with an alanine codon; AAA at position 491-493 was changed into CGT replacing the lysine codon with an alanine codon. These mutations created *Not* I site (underlined). The fragments were gel purified, mixed and subjected to another PCR amplification using T7 and SP6 primers, under the conditions described above, generating a band of 1.9 kb that was digested with *EcoR* I and after gel purification subcloned in *EcoR* I cut pCDNA3 plasmid. The orientation of the insert was determined by *Not* I digestion and the presence of the mutation was verified by sequencing. Double amino acid substitution mutant, HSF1:K224A/R227A was made by generating two PCR fragments, both on C12A plasmid as a template. The first, 800 bp long, was made with SP6 specific and NLSmut2 primer, corresponding to residues 222-228 (5'-GTACTGTGCACCATACG**CGGG**-3') and the second, 1.1 kb long, was generated with T7 and NLSmut1 primer corresponding to residues 223-231 (5'-CCCGCGTATGGTGCACAGTACTCCCTG-3'). The codon changes (shown in bold) were the following: A at positions 809 and 810 was changed into G and C respectively, replacing



the codon for lysine with an alanine codon and creating a *BstU* I site; C at position 818 was changed into G while G at position 819 was changed into C, replacing the codon for arginine with an alanine codon and destroying a *Sal* I site. The fragments were after gel purification mixed and subjected to PCR amplification using T7 and SP6 primers and the protocol described above. The resulting band of 1.9 kb was restricted with *EcoR* I and after gel purification subcloned into *EcoR* I digested pCDNA3. The mutations were verified by sequencing.

## Generation of mycPyruvateKinase-HSF1 Fusion (mycPK-HSF1)

A region of HSF1 corresponding to residues 198-230 was PCR amplified from C12A plasmid using an upstream primer, NLSup, corresponding to residues 198-202 (5'-GGGGTACCAGTCGAACCGGATCA-3') and a downstream primer NLSlow, corresponding to residues 225-230 (5'-GGGGTACCTAGGAGTACTGTCGACCATA3'). Both primers contained a *Kpn* I site at the 5' end (underlined) and the downstream primer also had an in-frame stop codon (shown in bold). The PCR fragment of 112 bp was restricted with *Kpn* I and after gel purification ligated to *Kpn* I linearized mycPK (Nakeilny *et al.*, 1995). The orientation of the insert was determined by digestion with the enzyme *BamH* I and the construct was verified by sequencing.

## Generation of dimerized GFP (GFP2) and of GFP2-HSF1 derivatives

GFP2 was generated by PCR on pEGFPC1 plasmid, using a pair of primers with a *Bgl* II site at 5' ends (underlined): primer GFPORFUP, corresponding to residues 1-8 of GFP open reading frame (5'-GAAGATCTATGGTGAGCAAGGGCGAGGAGCTG-3') and primer GFPORFDOWN, corresponding to nucleotides 1384-1407 from pEGFPC1 polylinker region (5'-GAAGATCTTCTAGATCCGGTGGATCCCGGGCC-3'). The resulting fragment of 730 bp was after *Bgl* II restriction and gel purification ligated to *Bgl* II restricted pEGFPC1 and the orientation was determined with *Ava* II digestion.

The pieces of HSF1 fused to GFP2 were generated by PCR on either C12A plasmid, (151-198 or 384-429) or on pCDNA1 containing HSF1: Δ160-172. They were subcloned in *Bam*H I site of GFP2. For fusion to GFP2 of HSF1 residues 151-198, the following primers were used: BamNESUp, corresponding to residues 151-158, (5'-CGGGATCCCAGGAGTGTATGGACTCCAAG-3') and BamNESDOWN, corresponding to residues 191-198 (5'-CGGGATCCCTACTGCACCAGTGAGATCAGGAACTG-3'). Both primers carried a *Bam*H I site at the 5' end (underlined) and BamNESDOWN carries an in-frame stop codon (shown in bold). These primers were used to generate a PCR fragment of 160 bp or of 120 bp if HSF1:Δ160-172 is used as a template. The fragments were cleaved with *Bam*H I, gel purified and subcloned into GFP2 linerized with the same enzyme. The orientation was determined by restriction with *Nhe* I and *Bst*X I. For fusion to GFP2 of HSF1 residues 384-429, the following primers were used: BamNESconUP, corresponding to residues

384-391 (5'-CGGGATCCCTGGATGCCATGGACTCCAACCTG-3') and BamNESConDOWN, corresponding to residues 422-429, (5'-CGGGATCCT ACTCTGCCTCAATAGGCCTGGGAGG-3'). Both primers carried a *BamH* I site at the 5'end (underlined) and BamNESconDOWN carries an in-frame stop codon (shown in bold). These primers generated a fragment of 150 bp that was cleaved with *BamH* I, gel purified and ligated to GFP2 linearized with *BamH* I. The orientation was determined by restriction with *Nhe* I and *Stu* I.

## Generation of GFP-NPc (Nucleoplasmin core) fusion constructs

A GFP fusion of NPc-TNLS (nucleoplasmin core followed by T antigen NLS) was created in the following way: myc-NPc-TNLS (Nakeilny *et al.*, 1995) was restricted with *BamH* I and *Xho* I and a 500 bp band, corresponding to NPc-TNLS piece, was purified. This was then ligated to pEGFPC1 cut with *Bgl* II and *Sal* I. Recombination of compatible *Bgl* II and *BamH* I and of compatible *Sal* I and *Xho* I sites keeps the reading frame in the resulting GFP-NPc-TNLS. The constructs GFP-NPc-TNLS-HSF1(151-198), GFP-NPc-TNLS-HSF1(151-198Δ160-172) and GFP-NPc-TNLS-HSF1(384-429) were generated by inserting NPc-TNLS in the corresponding GFP2 fusion construct from which the second copy of GFP was removed with *Bgl* II and *Sal* I. Recombination of compatible *Bgl* II and *BamH* I and of compatible *Sal* I and *Xho* I sites keeps the reading frame in the resulting constructs.

## **DNA Sequencing**

DNA sequencing was done by lab technicians Hanne Jorgensen and Monica Rimoldi using dideoxynucleotide chain termination method essentially as described in Pharmacia Biotech Sequencing Manual (1994).

## **PROTEIN DETECTION AND MANIPULATION**

### **Whole Cell Extract (WCE) Preparation**

Whole cell extract (WCE) was made essentially as described by Zimarino *et al.*, 1990, with some modifications. For harvesting the cells, the medium was discarded, the cultures were washed twice with PBS after which they were scraped in a small volume (up to 2 ml) of PBS using a disposable cell scraper (Costar). Cell suspension was transferred to a 2 ml eppendorf tube and quickly pelleted in microfuge for 15 sec at 13000 rpm. The supernatant was aspirated and cell pellet quickly frozen in liquid nitrogen or dry ice/ethanol. The pellet was stored at -80°C or immediately processed. WCE was prepared by thawing the pellet on ice in the presence of 3-5 volumes of extraction buffer (10 mM HEPES pH7.9; 400 mM NaCl; 1 mM EGTA; 5% glycerol; 0.1% TritonX-100). Prior to use 0.5 mM DTT, 0.5 mM PMSF, 1µg/ml of both pepstatine and leupeptine and 2.5 µg/ml of aprotinin (all from Boehringer) were added. The cell lysate was homogenized by repeated suspensions in a 200 µl micropipette tip and incubated on ice for 15 min. When WCE from RAT12 clone36 was made, homogenate was incubated on ice-water with stirring for 30 min. The homogenate was then transferred to thick-wall

polycarbonate tube (Beckman) and centrifuged for 15 min at 35000 rpm (100,000xg) at 4°C in Beckman ultracentrifuge. The supernatant (S100) was quickly frozen in liquid nitrogen and stored at -80°C.

### **Protein Concentration Determination**

Determination of proteins contents in WCE were done by the Bio-Rad Protein assay (Bio-Rad), also known as the Bradford assay, according to manufacturer's protocol. After the reaction mixture was assembled, OD750 nm was read in the spectrophotometer (Pharmacia) and protein concentration determined from a freshly prepared standard curve using appropriate dilutions of bovine serum albumin, (BSA) (Bio-Rad). The concentration of proteins was usually 3-8 mg/ml.

### **Sodiumdodecylsulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE was made essentially as described (Laemmli, 1970). The gels were poured in 14X16cm glass plates at ambient temperature. Separating gel mixture (20 ml), containing either 10% or 12% acrylamide, (taken from a stock solution with 29.2% acrylamide and 0.8% N,N'-methylene-bis-acrylamide), 375 mM Tris-HCl pH8.8 and 0.1%SDS was polymerized by addition of 0.1% ammonium persulfate (APS) and 0.05% N,N,N',N'-Tetramethylethylenediamine (TEMED). The stacking gel mixture (5 ml), containing 3% acrylamide, 125 mM Tris-HCl pH6.8 and 0.1% SDS, was polymerized by adding 0.1% APS and 0.05% TEMED. Protein samples, were boiled for 5 min at 100°C in 1X Laemmli sample buffer (2% SDS;

10% glycerol; 50 mM Tris-HCl pH6.8; 100 mM DTT; 0.005% bromphenol blue) and briefly spun for 1min at 13.000 rpm in microfuge. The gel was run at ambient temperature in 1X Tris-glycine buffer (25 mM Tris base; 250 mM glycine pH8.3; 0.1% SDS) under constant current of 30 mA, using Bio-Rad Electrophoresis Power supply.

### **Semi-Dry Electrophoretic Transfer**

Semi-dry electrophoretic transfer was performed essentially as described by Harlow and Lane, 1988. After the end of the run, the gel was placed on three pieces of 3MM gel blotting paper (Schleicher&Schuell) of the gel size soaked in semi dry transfer buffer (48 mM Tris base; 39 mM glycine; 0.037% SDS; 20% methanol). On top of the gel a piece of nitrocellulose transfer filter (Schleicher&Schuell; pore size 0.2  $\mu$ m) soaked in water was placed, followed by three pieces of gel blotting paper soaked in transfer buffer. The transfer was performed for 2-3 hours at room temperature in the Semi-Phor Transfer Apparatus (Hoefer Scientific Instruments) under constant current 0.8 mA/cm<sup>2</sup>. After the transfer, the filter was air dried for 15 min and processed for immunoblotting.

### **Immunoblotting**

The immunoblotting was performed using the protocol of Harlow and Lane (1988) with some modifications. The blocking, subsequent incubations with primary and secondary antibodies and washes of the filters were all performed at ambient temperature, except that the incubation with anti-GFP antibody was done at 4°C. The filter was

blocked for 1 h in 1:10 dilution of Blocking solution (Boehringer) or 5% non-fat dried milk, both diluted in TBST pH8 (100 mM Tris-HCl pH8.0; 250 mM NaCl; 0.1% Tween-20). The primary antibodies were used at the following conditions: rabbit anti-GFP (Clontech) at dilution 1:1000 in TBST pH8 overnight; rabbit anti-HSF1 (Fiorenza *et al.*, 1995) diluted 1:5000 in TBST pH12 for 1 h (TBST12 was freshly made from TBST pH8 by adjusting pH to 12 with 5M NaOH); mouse9E10 ascites fluid (anti-myc, gift from Angelo Corti), diluted 1:10.000 in TBST pH8 for 4 h and anti- $\alpha$  tubulin antibody (1:1000, Santa Cruz) for 4 hours in TBST8. Primary antibody incubations were followed with three washes, each for 15 min, in TBST pH8, except with TBST pH12 in case of the filter incubated with anti-HSF1. Secondary antibodies were the following: goat anti-rabbit IgG-HRP (Bio-Rad) and anti-mouse IgG-HRP (Boehringer), both diluted 1:10.000 in the blocking reagent (Boehringer). After the incubation with the secondary antibody, the filter was washed with TBST pH8 three times, 15 min each. The blots were developed with Chemiluminescence Western Blotting Kit (Boehringer) according to manufacturer's manual and exposed to KODAK X-OMAT films (Sigma).

## Gel Filtration

Gel filtration of proteins was done essentially as described by Farkas *et al.*, 1998, using the SMART system (Pharmacia) and Superose 6 PC 3.2/30 column (Pharmacia). WCE S100 supernatants (up to 40  $\mu$ l) containing 100-150  $\mu$ g of total proteins, were injected into the column calibrated, as described by Farkas *et al.*, 1998, with Blue Dextran, Acetone, Carbonic Anhydrase (27kD), Ovalbumin (43kD), Bovine Serum Albumin (BSA) (66kD), Alcohol Dehydrogenase (150kD), Beta-Amylase (200kD),

Apoferittin (443kD) and Thyroglobulin monomer (669kD) and dimer (1,338kD). Chromatography was done at ambient temperature (25°C) in the gel filtration buffer containing 20 mM Tris-HCl pH7.4; 140 mM NaCl; 10% glycerol; 1 mM EDTA; 0.2% TritonX-100; 0.5 mM DTT and protease inhibitors pepstatine 3 µg/ml, leupeptine 3 µg/ml, aprotinin 5 µg/ml and PMSF 1.5 mM. Before loading the sample, the column was equilibrated in the same buffer for 30 min. The following parameters were programmed: injection time of the sample from the sample loop to the column, flow rate (40 µl/ml) and fraction size (50 µl). After the fractions were collected, 17 µl of 4X Laemmli sample buffer and DTT to a final concentration of 100 mM were added and the samples were processed for SDS-PAGE analysis as described above.

## DNA-PROTEIN INTERACTIONS

### Gel Mobility Shift Assay

DNA-protein binding assays were done by the protocol described by Zimarino and Wu, 1987, using a consensus heat shock element (HSE) probe from a *Drosophila* hsp70 gene modified to contain 3 inverted repeats of NGAAN (Wu *et al.*, 1987). The 40 bp HSE probe (upper strand: 5'-GTCGACGGATCCGAGCGCGCCTCGAATGTTCTAGAAAAGG-3', with nGA An repeats underlined) was labeled with <sup>32</sup>P by primer extension as described by Farkas *et al.*, 1998 in the following way: The primer of 28 bp was annealed to the HSE 40-mer and 12 bases overhang filled in with Klenow enzyme. 2x10<sup>-8</sup> mM of annealed oligos were mixed with 30 mM of each dATP, dGTP and dTTP in a 30 µl reaction in 1.5 ml eppendorf



tube in 1X labeling buffer (50 mM Tris-HCl pH7.5; 10 mM MgCl<sub>2</sub>), 0.1 mCi of dCTP and 5U of Klenow DNA Polymerase (Boehringer) were then added and incubation continued for 30 min at room temperature after which the reaction was chased for next 10 min by adding dCTP to a final concentration of 30 mM. The reaction was stopped with 30 µl of 2X stop solution (20 mM EDTA pH8.0; 1% SDS) and after 40 µl of TES (10 mM Tris-HCl pH8.0; 1 mM EDTA pH8.0; 100 mM NaCl) was added, the labeled probe was purified on a NAP5 column (Pharmacia), pre-equilibrated in TES buffer, using manufacturer's instructions. The final concentration of labeled HSE probe in TES buffer was 30 nM. Binding mix (20 µl) contained 20 µg of WCE, 10 µg of poly(dI.dC)-poly(dI.dC) (Pharmacia) and 400 fmol of <sup>32</sup>P-labeled HSE in binding buffer (10 mM HEPES, pH7.9; 10 mM KCl; 50 mM NaCl; 1.5 mM MgCl<sub>2</sub>; 10% glycerol). In the case when competition was performed, 2 µl (2 pmol) of non-labeled primed HSE was added to the binding reaction, resulting in the 58X molar excess of non-labeled HSE. The volume up to 20 µl was adjusted with protein extraction buffer resulting in 130 mM final salt concentration. The binding was performed on ice for 20 min before 5 µl of 6X loading buffer was added and the sample loaded on the gel. Electrophoresis was done in 1% agarose gel in 0.5X TBE buffer (45 mM Tris-borate pH8.3; 1.5 mM EDTA) at ambient temperature and constant voltage of 60V for 2.5 h. The gel was capillary blotted onto DE81 paper (Whatman) overnight and the paper was autoradiographed in cassettes with intensifying screens (Kodak X-Omatic) at -80°C using KODAK films.

## CELL CULTURE AND MANIPULATION

### Growth Conditions for Tissue Culture Cells

The mammalian cell lines used in this study were the following: HeLa (human epithelial cell line, kind gift of Claus Nerlov), G2b2 (NIH 3T3 mouse fibroblast cell line, stably expressing bacterial  $\beta$ -galactosydase, kind gift of Olivier Bensaude), HSF1-/- (mouse embryonic fibroblasts derived from HSF1 knock-out mouse, McMillan *et al.*, 1998), kindly provided by Ivor Benjamin and RAT12 (rat fibroblast cell line stably expressing TetR/VP16 as described by Resnitzky *et al.*, 1994), kind gift from Kristian Helin. All cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), Penstrep (100 mg/ml) and Glutamine (2 mM). Amphotericin B (Fungizone) was added to the medium at concentration of 2.5 mg/ml. All cell culture reagents were purchased from GIBCO-BRL. The cells were cultured at 37°C under a 5% CO<sub>2</sub> atmosphere. Usually, medium was replaced every two to three days. The cells were grown in either 25 cm<sup>3</sup> (with 5-7 ml of DMEM) or 75 cm<sup>3</sup> (with 20-25 ml of DMEM) tissue culture flasks (BectonDickinson) and passaged after reaching confluency. For passaging the cells, the medium was discarded, cells were washed with the equal volume of Phosphate buffer saline (PBS, 200 mM NaCl; 20 mM KCl; 10 mM Na<sub>2</sub>HPO<sub>4</sub>; 1 mM KH<sub>2</sub>P0<sub>4</sub>), and cells were disattached by adding 1X Trypsin-EDTA solution and incubating for 5 min at 37°C. After incubation the cell suspension was transferred to 50 ml Falcon tube (Becton Dickinson) and collected by centrifugation at room temperature for 5 min at 2000 rpm. The trypsin solution was discarded and the cells were resuspended in appropriate volume of DMEM prewarmed at 37°C.

For freezing the cells, DMSO (Sigma) was added to the cell suspension in DMEM at the final concentration of 10%. The suspension was then transferred to a cryovial and put at -80°C for an overnight after which was transferred to a liquid nitrogen tank. The experiments described were done with exponentially growing cells.

### **Isolation of the cell nuclei**

Isolation of the cell nuclei was performed essentially as described elsewhere (Spector *et al.*, 1998). Rat12 cells, growing in tissue culture flasks, were trypsinized and collected as above. The cell pellet was washed with ice-cold PBS followed by resuspension in 5 volumes of ice-cold Nuclear Isolation Buffer (NIB; 60 mM KCl; 300 mM sucrose; 15 mM NaCl; 5 mM MgCl; 15 mM Tris-Cl pH 7.4; 0.5 mM DTT and 0.1 mM PMSF) and homogenization on ice in 1 ml Wheaton glass homogenizer with 10-15 strokes. The nuclei, pelleted in microfuge for 20 sec at 4°C, were resuspended in NIB buffer, incubated on ice for 10 min and pelleted as above. The nuclear pellet resuspended in 1 ml of NIB buffer was then overlaid on 9 ml of 1.5 M sucrose cushion in NIB buffer and nuclei were cleared by centrifugation for 30 min at 28,000 rpm at 4°C in Beckman Ti 50 rotor. Finally, the nuclear pellet was resuspended in a small volume (50  $\mu$ l) of NIB buffer and nuclei were attached to the glass coverslips by centrifugation for 5 min at 3000 rpm at room temperature in cytofuge. The coverslips were air dried for 10 min before immunostaining.

## The treatment with energy poisons

The inhibitors of ATP synthesis used were 10 mM sodium azide, 25  $\mu$ M oligomycin and 25  $\mu$ M antimycin (all Sigma), which were added to glucose minus DMEM (GIBCO BRL) supplemented with 6 mM 2-deoxyglucose (Sigma) as described (Guichon-Mantel *et al.*, 1991). The DMEM was also supplemented with 10% dialyzed FBS and glutamine and penstrep as described above. The RAT12 cells clone 36 (see below) growing on coverslips for 24 hours in the absence of tetracycline, were incubated at 37°C in DMEM containing energy poisons for indicated period of time (4 hours in case of sodium azide or 1 hour in case of oligomycin and antimycin; the treatment with oligomycin or antimycin was performed for 1 hour because prolonged incubations with these drugs provoked cell death). After that they were either fixed or washed thoroughly in PBS and put into the regular DMEM for additional 4 hours at 37°C and then fixed and stained with anti-HSF1 antibody. Identical results were obtained whether the protein synthesis inhibitor, cycloheximide (Sigma) at 100  $\mu$ g/ml was present or not.

## Transfections

HeLa cells were transfected with indicated expression plasmid using cationic lipid reagent Polyethyleneimine (PEI, Sigma) as described by Boussif *et al.*, 1995, with some modifications. 24 hours before transfection, 50.000 HeLa cells were seeded on a 13 mm glass coverslip (BDH) in a 24 multiwell plates with 1-2 ml of DMEM per well. The transfection mix was made at room temperature in 1.5 ml eppendorf tubes, as follows: 100  $\mu$ l of 150 mM NaCl were mixed with 6  $\mu$ l of 10 mM

PEI (made as aqueous solution from 5.5 M stock solution after which pH was adjusted to 7 with HCl), vortexed and spun in the microfuge for 15 sec. The mixture was then incubated for 2 min and then 2  $\mu$ g of plasmid DNA and 100  $\mu$ l of 150 mM NaCl were added; the mixture was again vortexed and briefly spun in the microfuge. The transfection mix was incubated for 15 min and then added to the cells. Multiwell plate was then centrifuged for 5 min at 1500 rpm after which the fine precipitate of PEI-DNA complex on the cells, could be observed under the microscope. The cells were then incubated at 37°C for next 3 h before the medium was aspirated, cells washed twice with PBS, trypsinized and pelleted as described above. The cell pellet was resuspended in DMEM, the cells were counted in hemocytometer and processed for cooling assay or cell fusions, as described bellow. Transfections for the immunoblots were done in 25 cm<sup>3</sup> flasks in which 1-1.2x10<sup>6</sup> HeLa cells were seeded 24 hours before transfection. Transfection mixture was made as described above, except that 50  $\mu$ g of plasmid DNA and 100  $\mu$ l of 10 mM PEI were used and the total volume was scaled up to 1 ml by addition of 150 mM NaCl. After the addition of the transfection mix, the cells in a flask were also centrifuged for 5 min at 1500 rpm. 3 h after transfection, the medium was discarded, replaced with 5 ml of fresh DMEM and the incubation at 37°C proceeded for additional 24 hours after which the cells were either subjected to heat shock and recovery treatment or collected and then processed for immunoblotting as described bellow.

## Generation of The Stable Clones Expressing HSF1

The stable transfectants expressing mouse HSF1 were made as follows: The plasmid Tet17 (1.8  $\mu\text{g}$ ) was cotransfected with 0.2  $\mu\text{g}$  of Puro-babe plasmid (Clontech), bearing Puromycin resistance gene in RAT12 cells using the protocol described above. After O/N incubation, the medium was replaced with fresh DMEM containing 500  $\mu\text{g}/\text{ml}$  of G418 (Sigma) and incubation was continued for another 48 hours. The cells were then replated in selective medium containing 0.5  $\mu\text{g}/\text{ml}$  of Puromycin (Sigma) and 1  $\mu\text{g}/\text{ml}$  of Tetracycline hydrochloride (Sigma) and cultured for two weeks changing the medium every two days. At this point, resistant cells were split in 96 multi wells dishes, having approximately 1 cell per well and clonal populations were derived after 3 weeks of culturing. The cell populations were then screened for the ability to express mouse HSF1 in the absence of Tetracycline by immunostaining (see below). 29 cell populations were screened (48 hours after Tetracycline withdrawal) and two of them (No. 5 and No.19) were found to containing approximately 1-2% of cells inducibly overexpressing HSF1. The population No. 19 was subjected to another round of limited dilution, resulting after additional 3 weeks in 25 clones, which were examined as above. Clones 36, 54 and 57 were all found to contain around 40-50% of cells overexpressing HSF1 at different levels upon Tetracycline removal (as judged by immunofluorescence staining). It should be pointed out that this heterogeneity in the content of HSF1 expressing cells, observed throughout procedure for making individual clones probably derives from having a mixture of cells, rather than individual cells in starting dilution. During this work it was observed that it was difficult to obtain single cell suspension of RAT12 cells since

attempt to make elutriation experiment with these cells failed due to the same reason. Therefore, clones rather represent a mixture of different cell populations which however does not affect interpretation of the results obtained using these cells. Secondly, in order to induce HSF1 expression, it was necessary to replate the cells in medium without Tetracycline rather than simply replacing the medium in the same flask. All the clones were maintained in DMEM containing 1  $\mu\text{g/ml}$  of Tetracycline (1000X Tetracycline stock solution, made in water was prepared every two weeks and stored at 4°C) and 0.5  $\mu\text{g/ml}$  of Puromycin (made as 2000X stock solution dissolved in water and stored at -20°C). Medium was replaced every 2-3 days during selection and maintenance of the stably clones. The clones have been since then passed through more than 20 passages and they all inducibly express HSF1. The clone 36 was chosen for subsequent experiments.

### **Heat shock and recovery treatments**

For heat shock of cultures in flasks or on coverslips in tissue culture dishes, medium was replaced with the same volume of fresh DMEM prewarmed at 42°C for 30 min and the cultures in flasks or the cells growing on the glass coverslips in 60/15 mm tissue culture dishes (Greiner), were submerged for 30 min in a 42°C water bath. For the recovery, after the initial incubation at 42°C, medium was discarded and replaced with a fresh DMEM, prewarmed at 37°C for 30 min and cultures in flasks or dishes were put at 37°C for indicated time period. In case of cultures in flasks, the cells were also gently shaken. After indicated times of heat shock or recovery the cells are both fixed and processed for immunostaining or collected for making protein extracts.

## **The cooling Assay**

The cooling assay was done with transiently transfected HeLa cells 24 hours post-transfection with either mycHSF1 or myc-NLS-PK (Ossareh-Nazari *et al.*, 1997) and with clone 36 cells 24 hours upon Tetracycline removal, using the protocol described by Michael *et al.*, 1995. Briefly, 50.000 cells of HeLa cells 3 hours post-transfection with indicated reporters (myc-NLS-PK or mycHSF1) or clone 36 cells, were seeded on 13 mm diameter CoverGlass (BDH) in 24 multi-well plate (Becton-Dickinson) with 1 ml of DMEM per well. Clone 36 was seeded in DMEM without tetracycline to induce expression of HSF1. After overnight incubation, the DMEM was replaced with the same amount of DMEM prewarmed at 37°C and containing 100 µg/ml of cycloheximide (Sigma). After 30 min of incubation at 37°C, medium was replaced with fresh DMEM (containing 100 µg/ml of cycloheximide), preequilibrated for 30 min on ice-water and the multi-well plates were put on ice-water for 20 min, after which incubation continued in a refrigerator at 4°C for indicated time periods (3, 4 or 6 hours). The cells were then fixed and processed for immunofluorescence as described.

## **Transient Interspecies Heterokaryon Assay**

Nucleocytoplasmic shuttling was detected by using a heterokaryon assay essentially as described previously (Guiochon-Mantel *et al.*, 1991), with some modifications. For making cell fusions, 100.000 of both fusion partners (HeLa cells and HSF1-/- mouse fibroblasts; clone 36 rat fibroblasts and HSF-/- cells; HeLa cells 3 hours post-transfection with appropriate reporter construct and G2b2 mouse fibroblasts) were seeded



on a 13 mm coverslip in a well of 12 multiwell dishes with 1-2 ml of DMEM per well. After overnight coculturing, DMEM was replaced with fresh DMEM prewarmed at 37°C for 30 min, containing 200 µg/ml of cycloheximide (Sigma) and the cells were incubated for 30 min at 37°C. The coverslips were then transferred to a tissue culture dish containing 20 ml of prewarmed PBS with 200 µg/ml of cycloheximide, briefly washed, the excess of PBS was drained off with a filter paper and a coverslip was put upside down on a drop (~ 8 µl) of 50% polyethyleneglycol, prewarmed for 30 min at 37°C (PEG; dissolved in PBS; Mol.Wt.3,000-3,700, Sigma) and placed on a piece of parafilm in a humidified chamber (90x16 mm tissue culture dish with a piece of filter paper soaked with water). Incubation was for 2 min at room temperature. The coverslips were then transferred to a Petri dish with 35 ml of PBS prewarmed for 30 min at 37°C, containing 200 µg/ml of cycloheximide and washed thoroughly around 2 min to remove all residual PEG, before being transferred to a 60/15 mm tissue culture dish with a DMEM prewarmed for 30 min at indicated temperature (37°C or 42°C in case of heat shock) and containing 100 µg/ml of cycloheximide. After the incubation at indicated temperature for the indicated time the heterokaryons were fixed and processed for indirect immunofluorescence as described below.

### **LeptomycinB Treatment**

LeptomycinB (LMB) was purchased from Novartis, it was dissolved in 96% ethanol as a 2 mM stock solution stored at -20°C and used essentially as described by Wolff *et al.*, 1997. For treatment of HeLa cells and heterokaryons, LMB was freshly diluted in DMEM as 4nM, 40 nM,

100 nM, 200 nM and 400 nM. For looking at the effect of LMB on subcellular localization of MAP kinase kinase (MEK1), HeLa cells were incubated overnight in DMEM containing 1% FBS; the medium was then replaced with DMEM containing 1% FBS and LMB in indicated concentrations for 2 hours in the presence of 100 µg/ml of cycloheximide, after which the cells were fixed with methanol/acetone and subjected to immunostaining. As a control, HeLa cells were in parallel treated with appropriate dilutions of absolute ethanol in DMEM under identical conditions. Treatment of heterokaryons with LMB included 2 hours of pretreatment of cocultured cells with indicated concentrations of LMB after which the cells were fused and incubation in LMB at indicated concentrations continued for additional 4 hours. Throughout all incubations cycloheximide was present at 100 µg/ml and DMEM was with 10% FBS. As a control, HeLa cells and the heterokaryons were treated with appropriate dilutions of absolute ethanol in DMEM. Another batch of LMB (Novartis), kind gift from Vincenzo Zapavigna (DIBIT HSR), made as a 10 mM stock solution in DMSO, was also tested as 40 nM, 100 nM, 200 nM or 400 nM dilution in DMEM (with corresponding dilutions of DMSO as the control) in treatment of heterokaryons giving identical results.

### **Heterokaryon Scoring**

The heterokaryon scoring was done by inspecting cell fusions under the microscope after immunofluorescence staining. For taking the heterokaryons into considerations two criteria were applied: first, cytokeratin,  $\beta$ -galactosidase or actin staining showing that the common cytoplasm is shared between two nuclei; secondly, Hoechst staining of

DNA showing that the heterokaryon was interspecies (1/3 of total fusion events). The extent of internuclear transfer was determined by giving arbitrarily the value of signal intensity in the recipient (mouse) nucleus that represent the percentage of signal intensity in donor (human) nucleus with apparent equilibrium being reach when the staining were of the same intensities. In case of fusions between HeLa and HSF1  $-/-$  cells, the time point at which an apparent internuclear equilibration was reached in around 50% of heterokaryons was used to calculate the minimal rate of export.

## **CELL STAINING**

### **Indirect Immunofluorescence and Immunofluorescence Microscopy**

Indirect immunofluorescence was done essentially as described by Harlow and Lane 1988, with some modifications. For doing immunofluorescence, the cells were grown on glass coverslips in tissue culture dishes or 12 (24) multi-well dishes having approximately 50-100.000 cells (200.000 in case of cocultures for making cell fusions) seeded on a 13 mm coverslip in a 12-multi well, day before immunostaining. Two protocols for cell fixation were used. In first, the cells were briefly washed with PBS and fixed with methanol/acetone (1:1) for 2 min, after which they were either immediately processed for blocking and immunostaining or kept in a sealed plastic container at 4°C for a maximum of few weeks. In the second protocol, the cells were fixed with 3% paraformaldehyde for 10 min, briefly washed twice with PBS and

permeabilized with 0.1% TritonX-100 for 5 min. During and after fixation, all incubations were done at room temperature. The coverslips were blocked in 3% BSA (Boehringer, dissolved in PBS and filtered through 0.2  $\mu$ m filter) for 1 hour after which the incubations with a primary antibodies, also for 1 hour, were done by inverting the coverslips upside down on a 8  $\mu$ l drop of antibody dilution on a piece of parafilm in humidified chamber (usually 90x16 mm Petri dish with a piece of paper soaked with water). The following antibodies, all diluted in 3% BSA, were used for immunostaining: rabbit anti-HSF1 (anti-whole molecule, 1:1000) (Fiorenza *et al.*, 1995); 10H8, rat monoclonal anti-HSF1 that recognizes an epitope in human HSF1 between amino acids 387-407 (1:100) (Cotto *et al.*, 1997); anti-cytokeratin 18 (Santa Cruz), mouse monoclonal, human specific (0.4  $\mu$ g/ml); anti- $\beta$ -galactosidase (Promega), mouse monoclonal (10  $\mu$ g/ml); rabbit anti-human MEK1 (1:1500), kind gift from Gilles L'Allemani; 9E10, mouse ascites fluid (a gift from Angelo Corti ), recognizing myc epitope (10  $\mu$ g/ml); rabbit anti-HSF1 (1:450), directed against carboxy-terminal peptide (LTGSEPPKAKDPTVS in single letter code) in human HSF1, kindly provided by Stuart Calderwood. Incubations with secondary antibodies, also diluted in 3% BSA, were done for 30 min in the same way like with the primary antibodies except that the samples were protected from light. Secondary antibodies used were the following: donkey anti-rabbit IgG-Rhodamine (10 ng/ml, Chemicon); sheep anti-mouse Ig-fluorescein, F(ab')<sub>2</sub> fragment (2  $\mu$ g/ml, Boehringer); sheep anti-rabbit IgG-fluorescein, F(ab')<sub>2</sub> fragment (2  $\mu$ g/ml, Boehringer); sheep anti-mouse Ig-Rhodamine, F(ab')<sub>2</sub> fragment (2  $\mu$ g/ml, Boehringer); donkey anti-rat Ig-Rhodamine (1  $\mu$ g/ml, Chemicon). After the incubations with the first and the second antibodies, the coverslips were transferred to 12 multi-well dishes and

washed three times, 5 min each, in 2 ml of PBS/0.1% TritonX-100 with gentle agitation. Staining of G actin with Phalloidin-fluoresceine (gift from Ivan De Curtis) was performed for 15 min, after the staining with the second antibody. The fluorescent dye Hoechst 33258 (Hoefer) was included in secondary antibody dilution at concentration of 1  $\mu$ g/ml. After the excess of washing buffer was drained off, the coverslips were mounted in a drop of Vectashield Mounting Medium (Vector Laboratories) on a 76x26 mm microscope slides (Menzel-Glaser), sealed with a transparent nail polish and stored in a closed plastic box protected from light at 4°C. The samples were observed with a 40x, 63x or 100x objectives with immersion oil under Zeiss Axiophot Microscope (Zeiss). The photos were taken using KODAK ELITE chrome 400 ASA film. The exposure times were determined empirically but they were usually in range of 30-90 sec for overexpressed proteins and 45-150 sec for endogenous proteins (for the fluoresceine and rhodamine filters and 15 sec for Hoechst filter in both cases). In some cases, the images were acquired using Hamamatsu digital camera C4742-95. Image elaboration was done using Adobe Photoshop software.

*In situ* competition assay was done by adding 10  $\mu$ l (approximately 200 ng as judged from a silver stained SDS gel) of bacterially expressed and chromatographically purified HSF1 or HSF2 proteins made by Karin Holm as described by Fiorenza *et al.*, 1995. The recombinant proteins were added during the anti-HSF1 incubation keeping the antibody dilution constant (1:1000).

## Determination of HSF1 stability in clone 36

For determining the stability of HSF1 in the absence of *de novo* protein synthesis, clone 36 cells stably expressing HSF1 were grown in the absence of tetracycline for 24 hours. After that, the protein synthesis inhibitor, cycloheximide (100  $\mu\text{g/ml}$ ) was added and incubation proceeded. At indicated time points starting from the cycloheximide treatment, the cell aliquots were collected by *in situ* lysis with RIPA buffer (150 mM NaCl; 50 mM Tris-HCl pH7.4; 1% SDS; 1% sodiumdeoxycholate) and the lysates were incubated on ice for 10 min in the presence of DNase I (10  $\mu\text{g/ml}$ ). The lysates were then cleared by centrifugation at 13000rpm for 10 min at 4°C after which they were processed for Western blot as described above.

## RESULTS

### Subcellular localization of HSF1 in unstressed and heat-stressed mammalian cells

The issue of subcellular distribution of inactive HSF1 in mammalian cells or its functional homologue HSF in *Drosophila*, has been for years a matter of controversy in the heat shock response field. Early studies have demonstrated that HSF DNA-binding activity can be induced *in vitro* by treating cytoplasmic extracts from unstressed mammalian and *Drosophila* tissue culture cells with heat and/or other stress conditions (e.g. low pH, urea), as well as with anti-HSF antibodies (Larson *et al.*, 1988; Mosser *et al.*, 1990; Zimarino *et al.*, 1990). Biochemical fractionation of mammalian and *Drosophila* cells, followed by immunoblot analysis showed that HSF1 was recovered in the cytosolic fraction from unstressed cells, but in the nuclear fraction of heat-stressed cells (Westwood *et al.*, 1991; Baler *et al.*, 1993; Sarge *et al.*, 1993; Zuo *et al.*, 1995; Mercier *et al.*, 1999). Despite some differences in fractionation methods employed, these results indicated that HSF1 is a cytoplasmic protein that is imported into the nucleus upon heat shock. This behaviour appeared similar to that of NF- $\kappa$ B (Henkel *et al.*, 1992), interferon regulatory factor 3 (IRF-3) (Yoneyama *et al.*, 1998) or hypoxia-

inducible factor-1 $\alpha$ , HIF-1 $\alpha$  (Kallio *et al.*, 1998), which in their latent, inactive form reside in the cytoplasm and inducibly translocate to the nucleus. However, contrary to the biochemical fractionation data, immunofluorescence staining of non-stressed, fixed mammalian tissue culture cells showed that HSF1 is localized either in both the cytoplasm and the nucleus (Sarge *et al.*, 1993), or predominantly in the nucleus (Rabindran *et al.*, 1991; Martinez-Balbàs *et al.*, 1995; Cotto *et al.*, 1997; Mercier *et al.*, 1999), whereas in heat-shocked cells it was always detected in the nucleus (Rabindran *et al.*, 1991; Sarge *et al.*, 1993; Martinez-Balbàs *et al.*, 1995; Cotto *et al.*, 1997; Mercier *et al.*, 1999). Similarly, results obtained by immunostaining of *Drosophila* cells showed that HSF is predominantly a nuclear protein in both non-stressed and heat-shocked cells (Westwood *et al.*, 1991; Orosz *et al.*, 1996). However, Zandi and coworkers (Zandi *et al.*, 1997) showed that *Drosophila* HSF is localized in the cytoplasm and translocates to the nucleus after heat shock. Finally, a very interesting observation came from Wang and coworkers (Wang *et al.*, 1998) demonstrating the existence of developmentally programmed nuclear relocalization in early *Drosophila* embryos, although the mechanism regulating this process remains unknown. Taken together, most of the immunofluorescence studies suggested that the bulk of HSF1, both in mammals and *Drosophila*, is constitutively nuclear. How can the discrepancy between the results of biochemical fractionation and cytological (immunofluorescence) assays be explained? Probably, it can be attributed to the difference



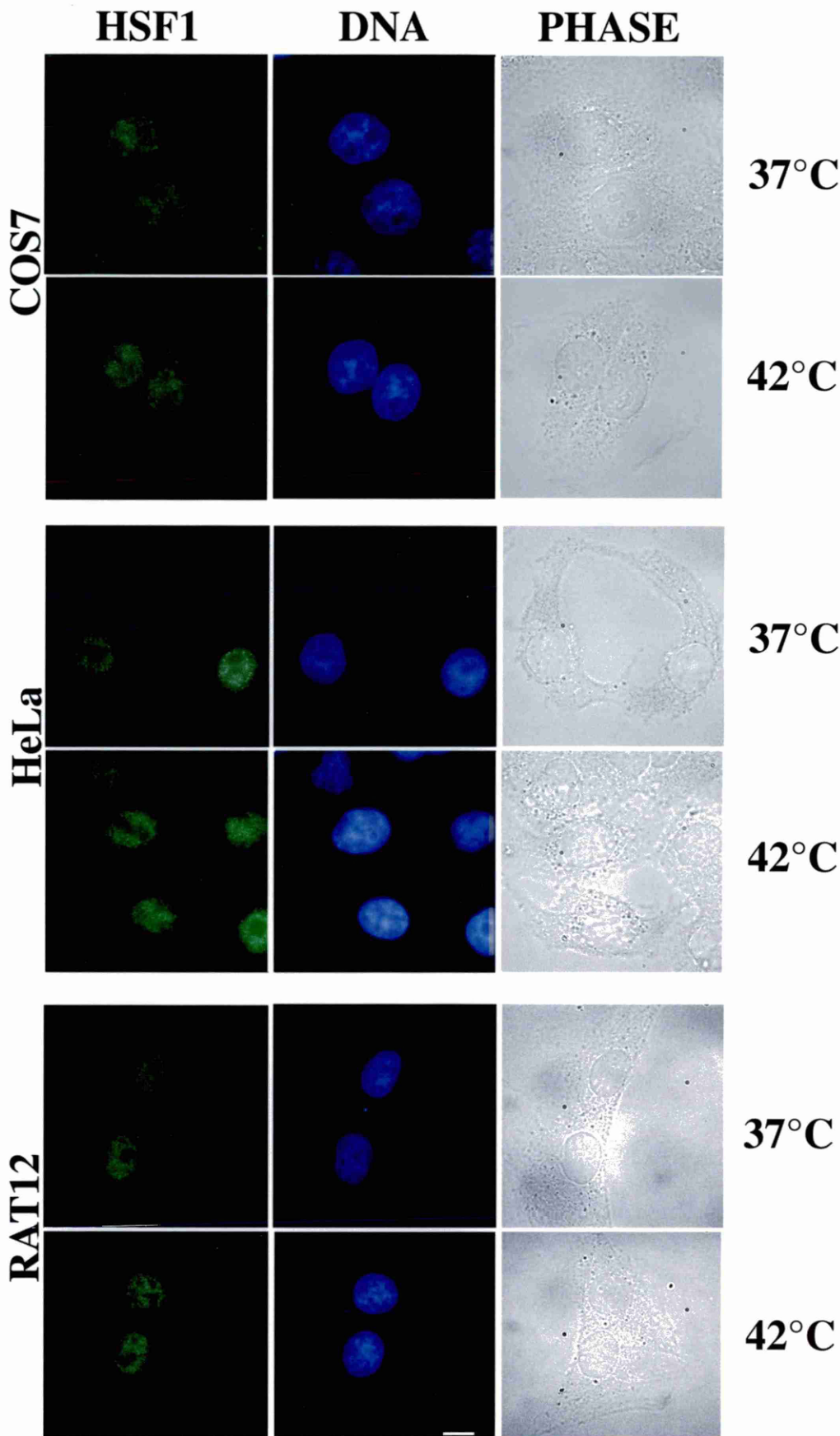
between the two methods and inevitable perturbation of cell integrity. Similar discrepancies have been shown previously for other proteins. For example, progesterone receptor and aryl hydrocarbon receptor are both shown by immunohistochemical analysis and cell enucleation to be predominantly nuclear, regardless of the presence or absence of ligands. However, upon cell fractionation these proteins were recovered in the cytosolic fraction (Jensen *et al.*, 1986; Perrot-Applanat *et al.*, 1985; Eguchi *et al.*, 1997). Others have observed that upon homogenization >50% of proteins are lost from the nuclei (Paine *et al.*, 1983), suggesting quantitative "leakage" of nuclear proteins during fractionation. This could explain why HSF1 is recovered in cytosolic fraction. On the other hand, the discrepancy between the results of immunofluorescence assays can not be explained in this way, but might reflect differences in the antibodies used, e.g. whether they were raised against denatured or native HSF1. Alternatively, distinct populations of HSF1 molecules may exist in unstressed cells and different antibodies may be more specific for one or the other. Also, different fixation procedures may have influenced antigenicity of HSF1.

Since the subject of this work has been to characterize inactive and stress-induced active forms of mammalian HSF1 at the cellular level, this issue required further clarification and was approached using a combination of cytological and biochemical methods starting with the indirect immunofluorescence analysis. To investigate subcellular localization of HSF1, several mammalian cell lines were

screened using a polyclonal antibody (anti-whole molecule) raised against recombinant mouse HSF1 produced in *E.coli* and purified under native conditions, as described by Fiorenza *et al.*, 1995. Cell lines examined were of monkey (COS-7), human (HeLa) or rat (RAT12) origin and in all of them HSF1 is found to be predominantly nuclear at normal growth temperature (Figure 6). As described previously for human HSF1 (Martinez-Balbàs *et al.*, 1995), in mitotic cells HSF1 was dispersed throughout, but excluded from chromosomes. In interphase cells, nuclear staining was homogeneous with apparent exclusion of nucleoli. The presence of HSF1 in the cytoplasm can not be ruled out, although, under the conditions used in this study cytoplasmic staining was not above general background level. Although it is possible that this antibody did not detect the cytoplasmic fraction of HSF1, results were similar when other anti-HSF1 antibodies were used (see below). In each case, the use of methanol/acetone or paraformaldehyde for fixation did not change the staining pattern.

When cells were shifted at 42°C for 30 min, (the condition that converts HSF1 to its active, DNA-binding form), the staining pattern remained unchanged in all cell lines examined (Figure 6). Conditions of mild and relatively short heat stress were preferred because they do not interfere significantly with cell physiology, yet they activate HSF1. It has been reported that upon heat stress, HSF1 in HeLa cells concentrates in discrete nuclear foci (Sarge *et al.*, 1993; Cotto *et al.*, 1997; Jolly *et al.*, 1997; Jolly *et al.*, 1999). These foci

Figure 6



**Figure 6. Nuclear localization of HSF1 in monkey (COS-7), human (Hela) and rat (RAT12) cells, maintained at 37°C or heat shocked at 42°C for 30 min.**

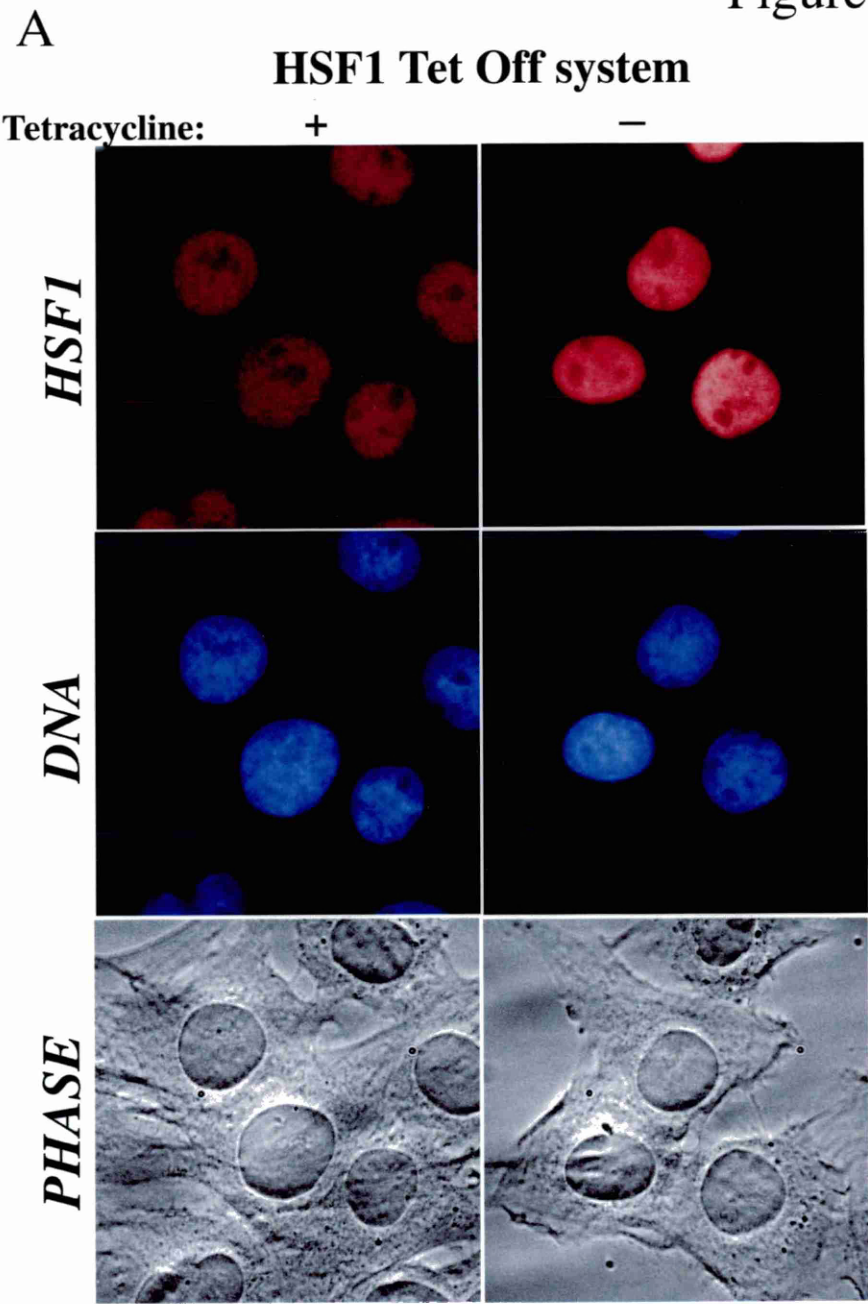
HSF1 was detected by indirect immunofluorescence with polyclonal anti-HSF1 antibody (anti-whole molecule), whereas DNA was stained with Hoechst. Scale bar = 5  $\mu$ m.

seem to be specific for primate cells and do not co-localize with other known nuclear structures. Moreover, they depend on the severity of the heat stress and can be induced also by other treatments (Cotto *et al.*, 1997). Heat shock conditions employed in this study did not result in HSF1 foci in HeLa or COS-7 cells presumably because they were not severe enough.

The same staining pattern was observed with affinity purified anti-HSF1 antibody (prepared by V.Zimarino), polyclonal antibody to denatured mouse HSF1 produced in *E.coli* (Sarge *et al.*, 1993), monoclonal antibody (Mab, epitope: 378-407, Cotto *et al.*, 1997) and a polyclonal antibody to a carboxy-terminal peptide of HSF1 (anti C-term end, Chu *et al.*, 1996). The specificity of antibody staining was also assayed by *in situ* competition: addition of native purified recombinant HSF1, but not of HSF2, completely abolished immunostaining, confirming that staining was specific for HSF1 (data not shown). Therefore, under conditions used in this study, HSF1 in mammalian cells is predominantly, if not exclusively, nuclear, both before and after heat stress. This implies that inactive HSF1 receives the heat stress signal in the nucleus.

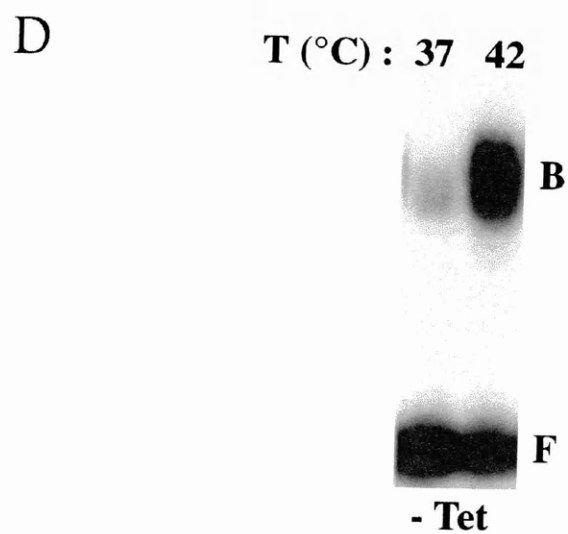
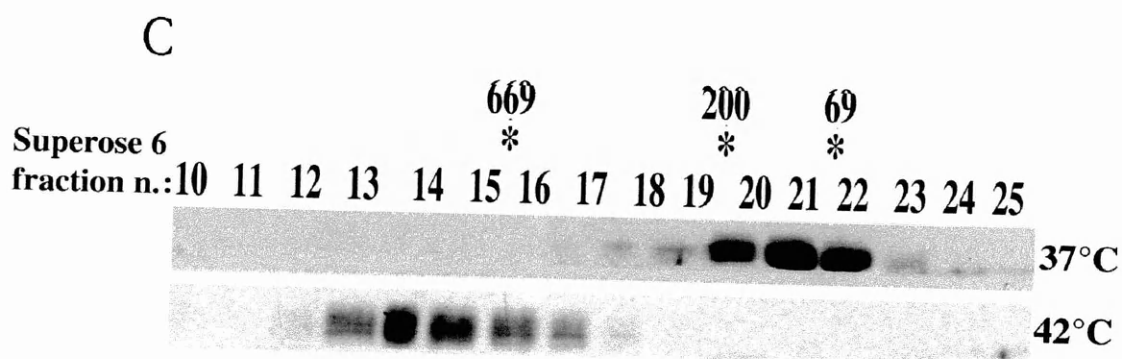
In RAT12 derivative clone 36, in which expression of mouse HSF1 is induced by tetracycline removal ("Tet-Off" expression system, Gossen and Bujard, 1992), the HSF1 staining was nuclear in ~95% of the cells (Figure 7A). In a small percentage of "Tet-Off" cells (~5%), a weak signal above background was also detected in the cytoplasm. On average, these cells express mouse HSF1 approximately 10 fold above

Figure 7



**B**

	RETIC	Rat12	cl.36 Tet		
			+	-	kD
					97
	[				68
					43
non specific	[				29



**Figure 7. Stable expression of mouse HSF1 in RAT12 fibroblasts in Tetracycline-regulated manner ("Tet-Off")**

- (A) Nuclear localization of HSF1 in clone 36 cells 24 hours after tetracycline withdrawal. HSF1 was detected by indirect immunofluorescence with anti-HSF1 antibody.
- (B) Immunoblot with anti-HSF1 antibody on protein extracts from parental RAT12 cells (RAT12), clone 36 cells grown in the presence of tetracycline (+Tet) or 48 hours after withdrawal of tetracycline (-Tet), and an aliquot of mouse HSF1 translated *in vitro* (RETIC).
- (C) Immunoblots showing Superose 6 fractionations of whole cell extracts from clone 36 cells maintained at 37°C or heat shocked at 42°C for 30 min. Peak elutions of protein markers thyroglobulin (669kD), beta-amylase (200kD) and BSA (69kD) are indicated above the lanes.
- (D) Electrophoretic mobility shift assay showing induction of the HSE-binding activity in heat-shocked clone 36 cells. B - bound probe; F - free probe.



the endogenous protein, as judged from the immunoblot analysis (Figure 7B). Importantly, gel filtration chromatography in a Superose 6 column showed proper regulation of the oligomeric state of HSF1 in this expression system. HSF1 extracted from cells at 37°C peaks in fractions n. 20-22, whereas HSF1 from heat shocked cells was recovered in the earlier fractions n. 13-16 (Figure 7C). These elution profiles showed the characteristic shift which is indicative of heat shock induced oligomerization of HSF1, i.e. monomer to trimer transition (Rabindran *et al.*, 1993; Farkas *et al.*, 1998). Both inactive and active forms of HSF1 chromatograph as larger species than expected: inactive HSF1 elutes as a species of ~100 kD, whereas active HSF1 elutes as a species of >600 kD. This is in agreement with previous observations which suggested that both monomer and trimer are non-globular and therefore produce anomalous measurements in gel filtration chromatography (Larson *et al.*, 1995; Farkas *et al.*, 1998). Consistent with the results of gel filtration, HSE-binding activity is induced in heat shocked cells (Figure 7D).

Taken together, these data show that HSF1 stably expressed in this inducible system is correctly regulated, since it adopts the behavior of the endogenous protein. These properties make the "Tet-Off" expression system suitable for studying regulation of HSF1, because inducible, moderate overexpression does not result in deregulation (i.e. constitutive oligomerization and DNA binding) as often seen in other transient or stable overexpression systems in which the level of HSF1 could not be controlled.

In an effort to look at the localization of HSF1 using a cell fractionation assay, nuclei were isolated from non-heat shocked and heat shocked RAT12 cells using hypotonic lysis of cells followed by purification of the nuclei through a sucrose cushion and immunostaining (see Materials and Methods). The results showed that the nuclei isolated from unstressed cells were not stained with anti-HSF1, while the nuclei isolated from heat-stressed cells stained positively for HSF1 (Figure 8A). The loss of HSF from non-shocked nuclei, which is most probably due to "leakage" through (damaged) nuclear membrane during fractionation, is in agreement with the results of a similar experiment performed on *Drosophila* SL-2 cells by Westwood *et al.*, 1991. They also showed that HSF is quantitatively lost from the nuclei isolated from non-stressed cells and is recovered in the cytosol. Similar observations have been made earlier with estrogen and progesterone receptor, which both reside in the nucleus (Perrot-Applant *et al.*, 1985; King and Greene, 1984), irrespectively of whether they are bound to the ligand or not. However, after homogenization, ligand-free receptors were found in the cytosol (Gorski *et al.*, 1968). Others, as mentioned above, observed that this phenomenon of "leakage" of nuclear proteins after homogenization is more general (Paine *et al.*, 1983).

However, nuclear residency of HSF1 can be perturbed reversibly in intact tissue culture cells thus mimicking the distribution of HSF1 during homogenization of non-stressed cells. For this purpose, clone 36 cells were treated with

Figure 8A

**IMMUNOSTAINING OF ISOLATED  
RAT12 CELL NUCLEI**

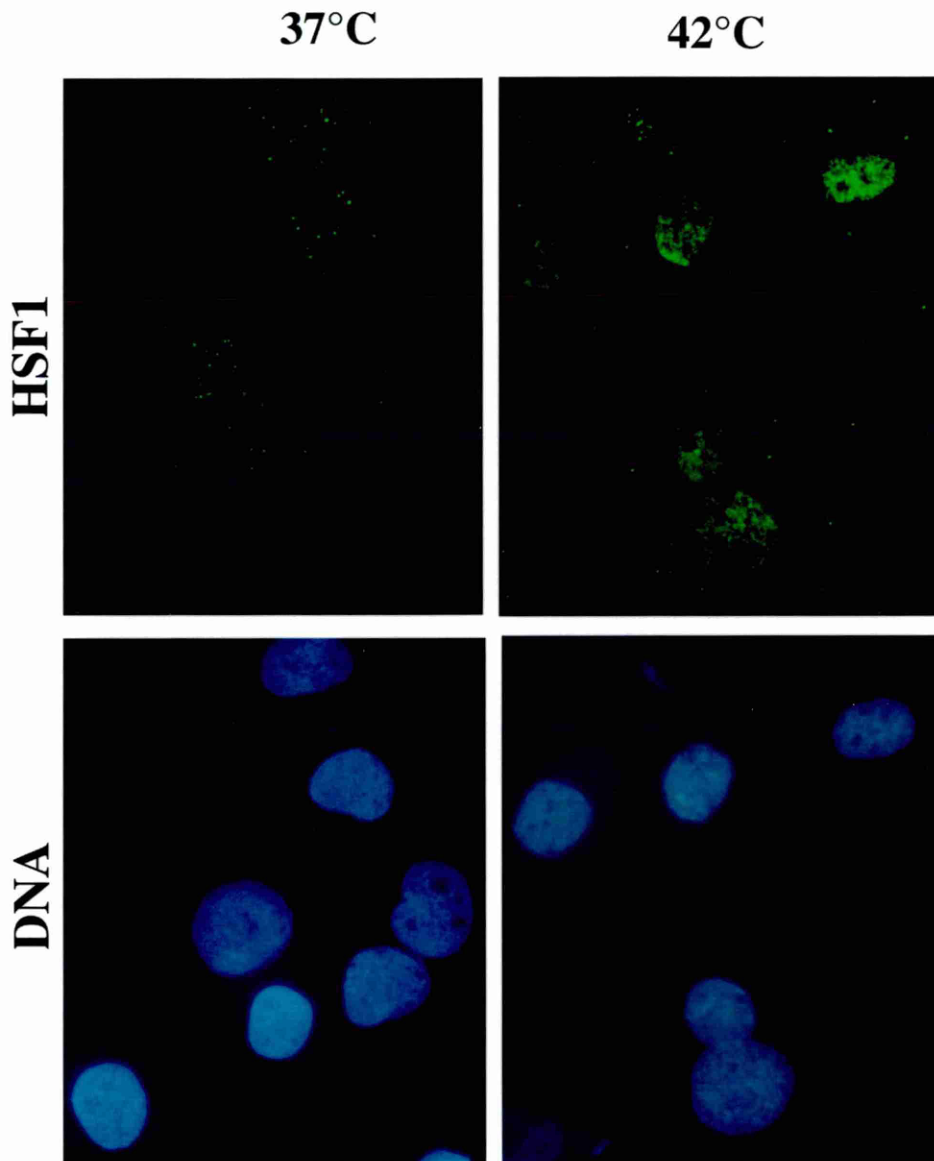
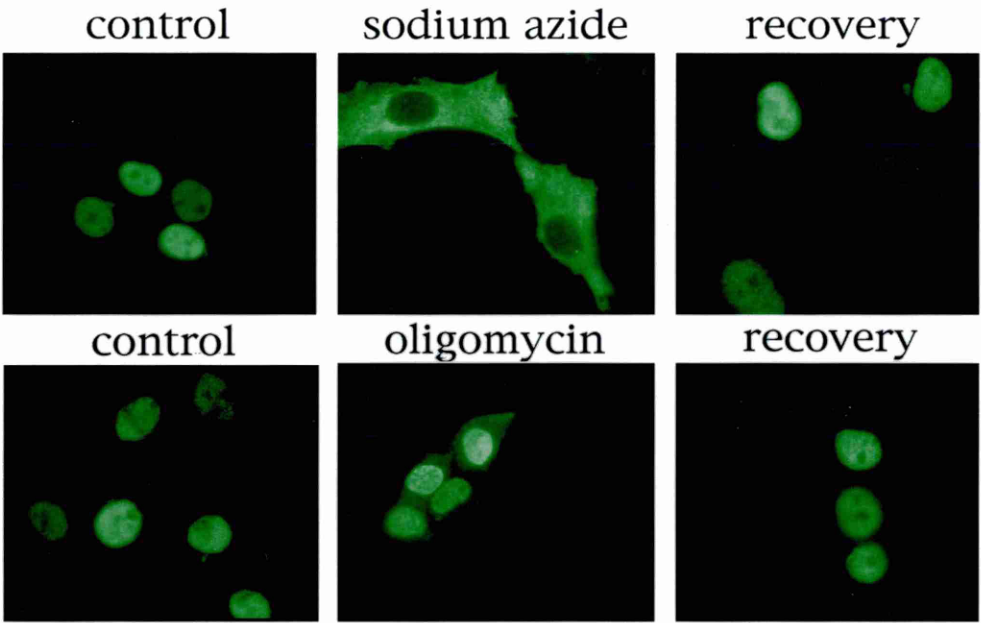


Figure 8B



**Figure 8. Subcellular fractionation and treatment with energy poisons provoke nuclear exit of HSF1.**

(A) HSF1 staining of RAT12 nuclei isolated from the cells maintained at 37°C or heat shocked at 42°C for 30 min.

(B) HSF1 staining of clone 36 cells: untreated (control), treated for 4 hours with 10mM sodium azide or for 1 hour with 25µM oligomycin. In parallel, the cells treated with indicated drugs were recovered for 4 hours in regular DMEM (recovery). All incubations were done in the presence of cycloheximide (100µg/ml).

various energy poisons. In the presence of cycloheximide to block *de novo* protein synthesis, energy poisons (inhibitors of ATP synthesis) sodium azide, oligomycin and antimycin, provoked extranuclear localization of HSF1 which was detected in a majority of cells after 4 hours of treatment with sodium azide or 1 hour of treatment with oligomycin or antimycin. Figure 8B shows representative staining of the cells treated with sodium azide or oligomycin, whereas Table 1 summarizes the results of scoring >100 cells for the effect of each. By visual inspection, three types of staining patterns were assigned to the cells: nuclear (N, as shown for control or recovery), predominant nuclear with a visible cytoplasmic component (N>C, as for oligomycin-treated cells) or predominant cytoplasmic (C>N, as shown for sodium azide-treated cells). Although the bulk of HSF1 remained nuclear, in the majority of cells treatment with energy poisons provoked appearance of the protein in the cytoplasm (N>C). The difference observed between cells treated with sodium azide and oligomycin or antimycin can be attributed to the variability in penetration of these drugs into the cells and to the fact that their mechanism of action is different, as reported previously (Klingenberg, 1989; Guiochon-Mantel *et al.*, 1991). In addition, the treatment with oligomycin or antimycin longer than 1 hour were lethal to the cells. Importantly, nuclear exit of HSF1 was, in all three cases reversible; when the drugs were washed out and cells put under normal conditions, nuclear localization of HSF1 was restored within 4 hours as shown in representative

TREATMENT	SUBCELLULAR LOCALIZATION		
	nuclear	nuclear& cytoplasmic	cytoplasmic
NONE	95%	5%	
SODIUM AZIDE (10mM)	10%	80%	10%
SODIUM AZIDE/ RECOVERY	95%	5%	
OLIGOMYCIN (25 $\mu$ M)		100%	
ANTIMYCIN (25 $\mu$ M)		100%	

**Table 1.** Summary of the effect of energy poisons on subcellular localization of HSF1 in "Tet-Off" clone 36 cells. Based on the immunostaining, the cells were categorized in three groups: nuclear staining, predominant nuclear with visible cytoplasmic staining and cytoplasmic staining. For each experimental point >100 cells were scored.

immunostaining (sodium azide/recovery) in Figure 8B. Since protein synthesis was blocked throughout the course of the experiment, this indicated that the same HSF1 pool was relocating to the cytoplasm and back to the nucleus. This suggests that nuclear residency of HSF1 may reflect a more dynamic situation: although at steady state highly asymmetrically distributed, HSF1 has the ability for bidirectional transport across the nuclear envelope. A similar observation was made for the progesterone receptor, which reversibly relocated to the cytoplasm in cells treated with energy poisons (Guichon-Mantel *et al.*, 1991). It was proposed that this shift in nucleocytoplasmic distribution could be due to the nuclear efflux of the receptor which, unlike nuclear import, occurs under the conditions of energy depletion. In addition, this effect depended on the NLS of the receptor suggesting that the NLS also mediates nuclear exit of this protein (Guichon-Mantel *et al.*, 1994). It is possible that the change in subcellular distribution of HSF1 in the cells treated with energy poisons is due to the same effect, but it is not known that any specific sequence in HSF1 is required. Since the effect of energy poisoning was seen with sodium azide, oligomycin and antimycin which all have different mechanisms of action, it is unlikely that their effect on subcellular distribution of HSF1 was due to a non-specific action of each of them. Moreover, exit of HSF1 from the nucleus can not be attributed to irreversible cell damage since the effect of energy poisons is reversible: when the cells



were transferred back into medium devoid of these drugs, HSF1 regained its initial maximal nuclear localization.

## Sequence requirements for the nuclear localization of HSF1

The next question that was addressed was whether the constitutive nuclear accumulation of HSF1 depends on the integrity of its conserved domains. A set of experiments were performed in which HSF1 bearing mutations in either the DNA-binding domain, trimerization domain (HR-A/B) or carboxy-terminal leucine zipper 4 (HR-C) was fused to the carboxy-terminus of green fluorescent protein (GFP) and transiently overexpressed in HeLa cells. 24 hours post-transfection, their subcellular distributions were examined under the microscope. The first mutation was a deletion in the DNA-binding domain of residues 6 to 80 ( $\Delta 6-80$ ) that abrogates DNA binding (Farkas T., 1995). The second mutation was a deletion in the trimerization domain removing residues 160-172 ( $\Delta 160-172$ ), making the protein unable to oligomerize (Farkas T., 1995). The third mutation was a single amino acid substitution in the trimerization domain; histidine at position 179 was replaced by arginine, H179R. This mutant was shown to be constitutively oligomeric when expressed *in vitro* (Farkas *et al.*, 1998). Finally, triple amino acid substitution M387K/L391A/L394A in the HR-C (leucine zipper 4) was also analyzed. This mutation, like H179R, relieves suppression of trimerization making the protein constitutively oligomeric *in vivo*, as shown previously (Rabindran *et al.*, 1993). Wild type (wt) HSF1 and each mutant were nuclear in at least 90% of transfectants, unlike

free GFP, which appeared distributed diffusely throughout the cell (Figure 9A). In each case, the same result was observed without GFP tag (data not shown). Therefore, functional integrity of conserved domains does not seem to be required for nuclear accumulation of HSF1. In addition, the GFP moiety did not appear to influence localization of HSF1. Figure 9B shows a protein blot probed with an anti-GFP antibody, confirming the predicted molecular mass of the polypeptides. The relevant GFP-HSF1 chimeras were analyzed by Superose 6 gel filtration chromatography to evaluate their native sizes. Protein extracts from HeLa cells 24 hours post-transfection were fractionated in Superose 6 column and the column fractions were assayed by probing protein blots with anti-GFP antibody. Figure 9C shows that GFP-HSF1<sub>wt</sub> adopted the proper regulation because 30 min heat shock at 42°C induced the expected increase of its native size. Moreover, when cells were first heat shocked and then allowed to recover at 37°C, GFP-HSF1 dissociated back to the monomeric form. Complete dissociation was observed after 3 hours of recovery, although it was clearly detectable already at 30 min (Figure 9C). These results were reproducible in many experiments. In contrast, GFP-HSF1:Δ160-172 did not show differences in native size between non-stressed and heat-shocked cells, (Figure 9C). Elution profiles of GFP-HSF1:H179R and GFP-HSF1:M387K/L391A/L394A were consistent with the expectation that they exist *in vivo* as oligomeric species at 37°C, although their elution profiles were not identical (Figure 9C).

Figure 9A

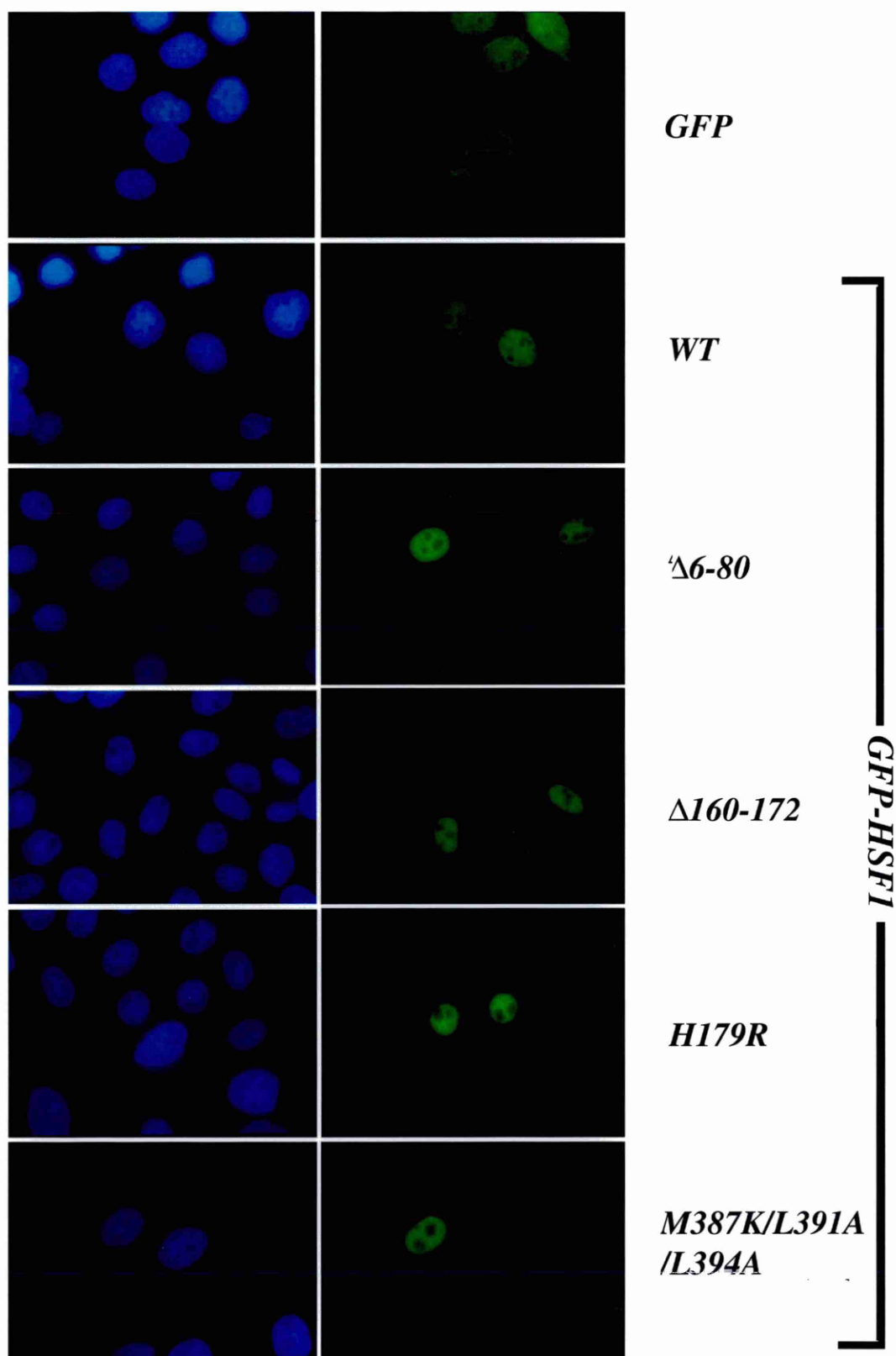
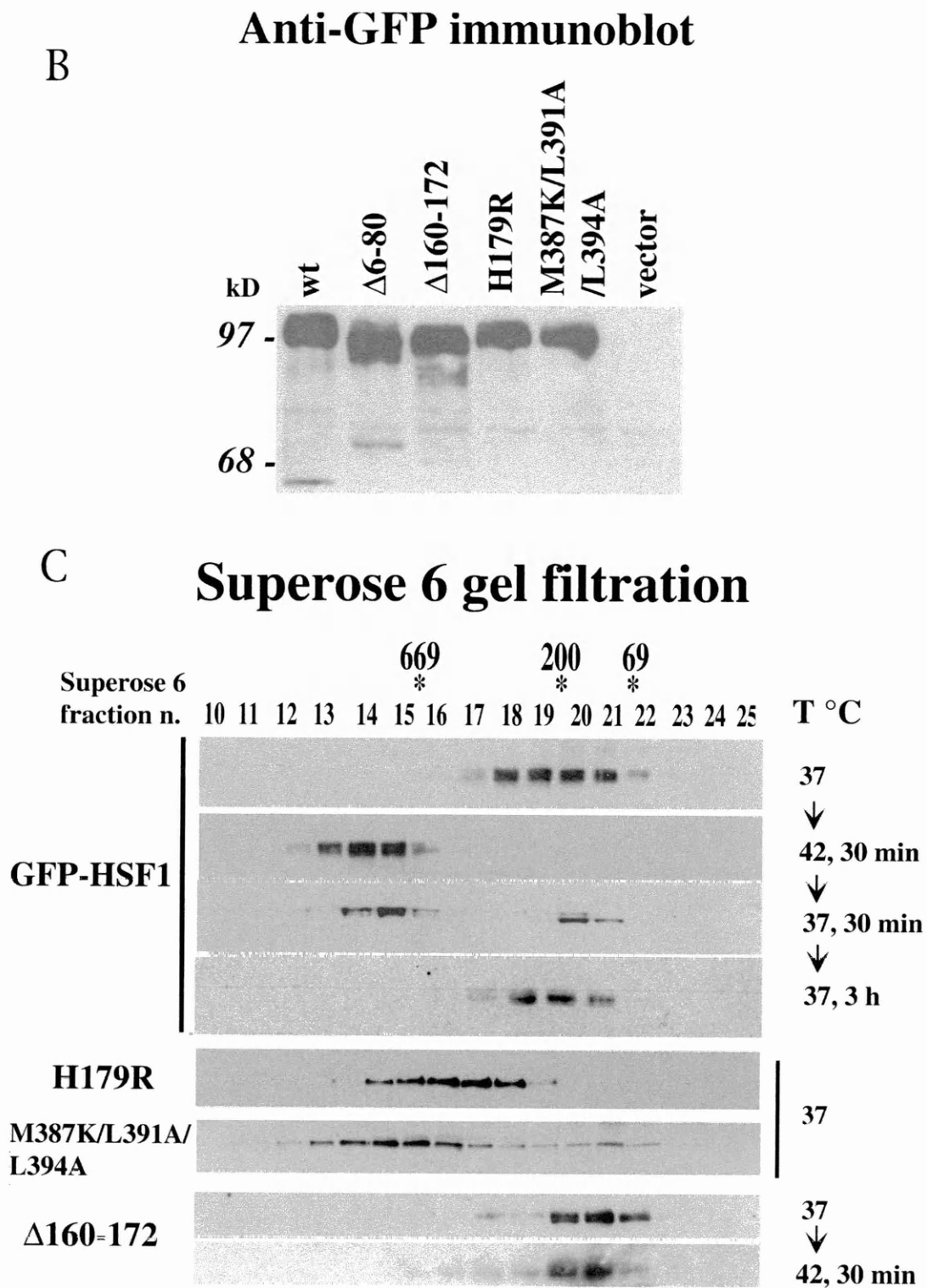


Figure 9



**Figure 9. Analysis of GFP-HSF1 chimeras after transient overexpression in HeLa cells.**

(A) Nuclear localization of GFP-HSF1 wild type (wt) and GFP-HSF1 mutants ( $\Delta 6-80$ ,  $\Delta 160-172$ , H179R and M387K/L391A/L394A). In contrast, GFP shows diffuse distribution.

(B) Immunoblot with anti-GFP antibody on whole cell extracts.

(C) Immunoblots showing Superose 6 fractionations of whole cell extracts.

In case of wt and  $\Delta 160-172$ , the original pool of transfectants was split into four or two portions, respectively, and the cells were either maintained at 37°C, heat shocked at 42°C for 30 min or heat shocked and then recovered at 37°C for indicated period of time (30 min or 3 hours). Peak elutions of protein markers thyroglobulin (669kD), beta-amylase (200kD) and BSA (69kD) are indicated above the lanes.

The above proteins showed comparable nuclear accumulation, leading to the conclusion that nuclear residency of HSF1 is not coupled to either its oligomeric state or sequence-specific DNA binding ability. Importantly, the behavior of these fusion proteins (summarized in Table 2) makes them reliable for further investigation of HSF1 at the cellular level.

Since the transport of proteins between the nucleus and cytoplasm through nuclear pore complexes (NPC) is in general selective and signal-mediated (Gorlich and Mattaj, 1996), a subtractive approach was employed in order to define minimal sequence requirements for nuclear accumulation of HSF1. For this purpose a series of carboxy-terminal deletions of mouse HSF1 tagged with the myc epitope at the amino-terminus has been constructed. Their subcellular distribution has been examined by immunofluorescence with anti-myc antibody after transient expression in HeLa cells (24 hours post-transfection). Figure 10 shows representative examples of immunofluorescence analysis of each mutant along with the corresponding histogram. For each histogram >300 transfected cells have been scored in three independent transfection experiments. After visual inspection four staining patterns were assigned: N, for nuclear only, N>C, for predominantly nuclear, but with visible cytoplasmic signal, N=C, for equal distribution between nucleus and cytoplasm and C>N, for predominantly cytoplasmic. As shown in Figure 10, progressive deletion of amino acids from the carboxy-terminus that include end points at residues 382 (1-382), 266 (1-266),

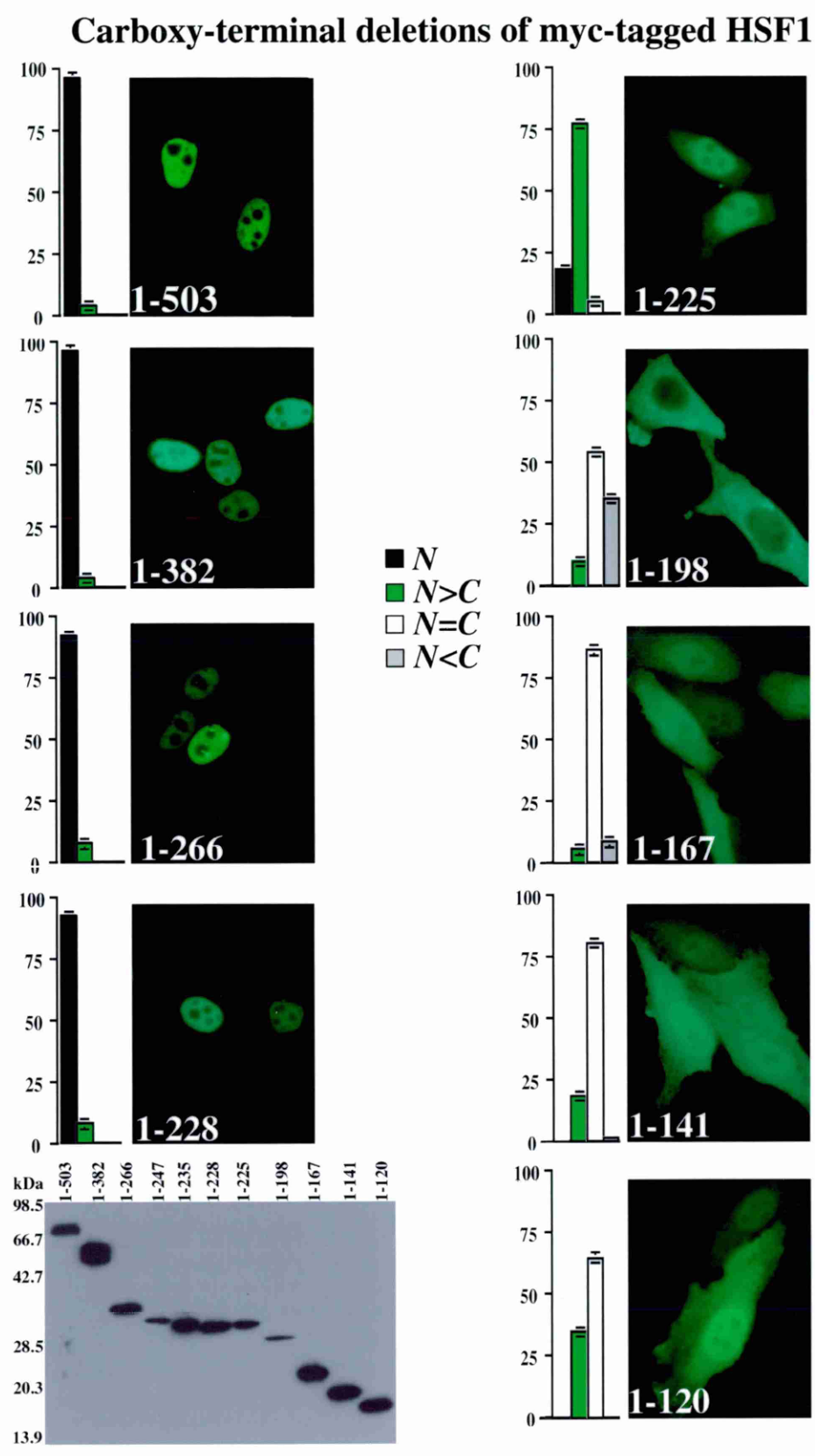
GFP-HSF1	localization 37°C and 42°C	oligomeric state	
		37°C	42°C
wt	nuclear	monomer	oligomer
Δ160-172	nuclear	monomer	monomer
H179R	nuclear	oligomer	n.d.
K387/A391/A394	nuclear	oligomer	n.d.

**Table 2.** Summary of the analysis of relevant GFP-HSF1 chimeras in transiently transfected HeLa cells. Subcellular localization was determined by fluorescence, whereas oligomeric state was assayed by gel filtration chromatography of whole cell extracts.  
n.d. indicates not determined.



247 (1-247), 235 (1-235) and 228 (1-228) did not alter the extent of nuclear accumulation observed with the full length HSF1 (1-503). Further deletion of 3 residues caused a prevalence of N>C, as shown in 1-225, suggesting that nuclear accumulation was not maximal. When residues between 225 and 198 were deleted, 1-198 was apparently equally distributed between the two compartments in the majority of cells. However, in >30% of cells 1-198 was predominantly cytoplasmic (C>N), as shown in the corresponding figure. Deletion mutants ending at positions 167 (1-167), 141 (1-141) and 120 (1-120) all showed N=C type of distribution. Figure 10 also displays polypeptide species detected with the anti-myc antibody. The results of this analysis indicated that a major sequence element for nuclear import resides between residues 198 and 228 (Figure 11A). Although HSF1 does not contain an easily recognizable "classical" NLS consensus, inspection of this region has revealed a cluster of three basic amino acids, lysine, arginine and lysine (KRK in single letter code), at position 206-208 that might be critical (Figure 11A). A classical SV40-type NLS usually contains one or more clusters of basic amino acids. For example, the NLS in the large T antigen of simian virus 40 (SV40) (Newmeyer *et al.*, 1986) contains 7 residues (PKKKRKV); the bipartite NLS of nucleoplasmin (Dingwall *et al.*, 1982) contains two basic clusters (underlined) separated by a 10 amino acid linker (KRPAAIKKAGQAKKKK). To address a possible role of clustered basic residues in HSF1 as a potential NLS motif, site-directed mutagenesis approach was used and the residues at positions

Figure 10



**Figure 10. Analysis of carboxy-terminal deletion mutants of myc-tagged HSF1 in transiently transfected HeLa cells.**

Subcellular localization of mycHSF1wt (1-503) and carboxy-terminal deletion mutants detected by indirect immunofluorescence with anti-myc antibody (9E10). The end point of each deletion mutant is shown on the figures. The corresponding histograms are derived from scoring >300 cells in three separate experiments. By visual inspection, four categories of subcellular distribution were assigned: nuclear (N), predominant nuclear with visible cytoplasmic portion (N>C), an equal distribution between two compartments (N=C) and predominant cytoplasmic staining (C>N).

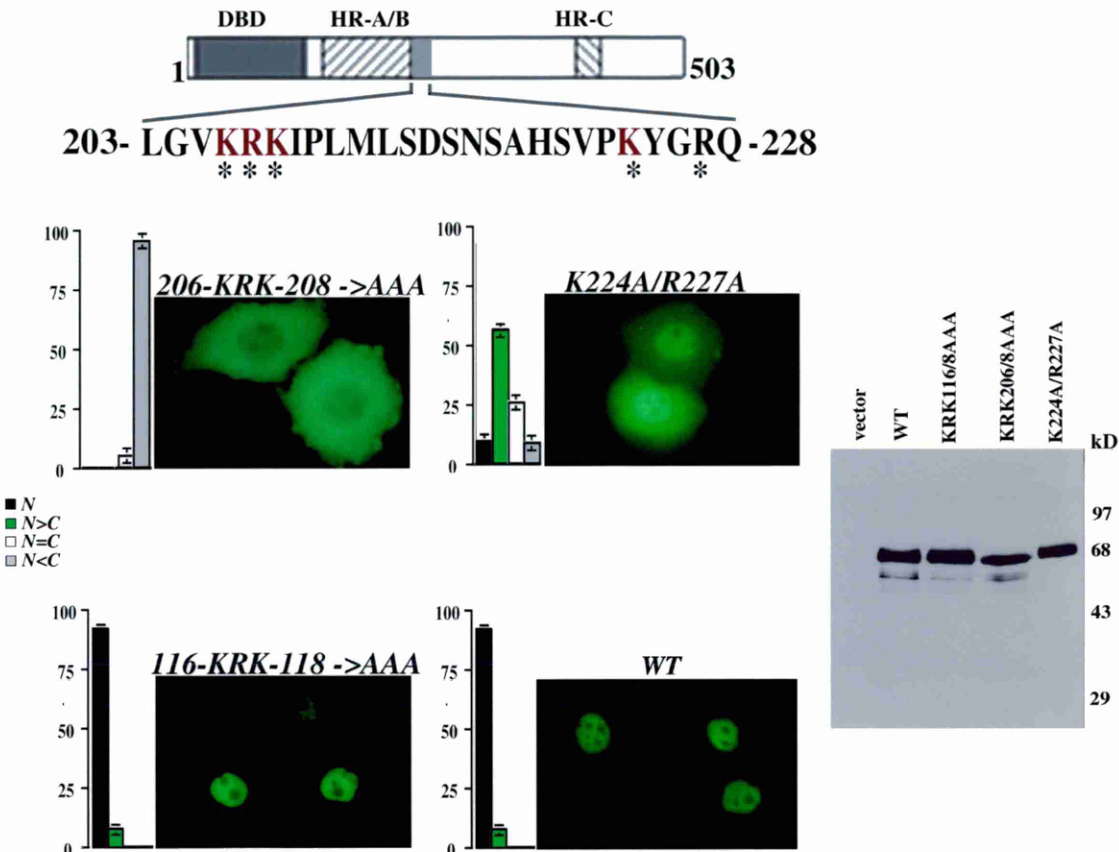
Below is shown immunoblot on whole cell extracts with anti-myc antibody.

206-208 were exchanged by alanines. The mutation was tested in the context of the full length HSF1 in transiently transfected HeLa cells. As shown in Figure 11A, HSF1:KRK206/8AAA was quantitatively relocalized to the cytoplasm. This demonstrates that basic cluster 206-KRK-208 is essential for nuclear targeting. However, as shown above, full nuclear localization also required a region located more to the carboxy-terminus, since 1-228 was fully nuclear unlike 1-225. By sequence inspection of the region 209-228, two basic residues were found: lysine at position 224 and arginine at position 227. These two residues were mutagenized to alanines and assayed in context of full length HSF1. As shown in Figure 11A, this mutation had a modest effect on HSF1 subcellular distribution, in comparison to KRK-206/8-AAA mutant. The bulk of HSF1: K224A/ R227A was nuclear; however, a significant amount of the protein was cytoplasmic (Figure 11A). Contrary to the previously published data (Zhuo *et al.*, 1995), mutation to alanines of another basic cluster, KRK at position 116-118 did not influence nuclear residency of HSF1 (Figure 11A). Polypeptide species encoded by all mutants had the expected molecular mass as judged by a protein blot probed with anti-HSF1 antibody (Figure 11A). Thus, two sequence elements are required for nuclear import of HSF1. The first comprises a cluster of three basic residues, KRK, at position 206-208 and has a major effect. The second, 15 residues apart, includes two non-clustered basic residues, K at position 224 and R at position 227, having a minor, but yet distinct role. It would be interesting to test the effect of

Figure 11

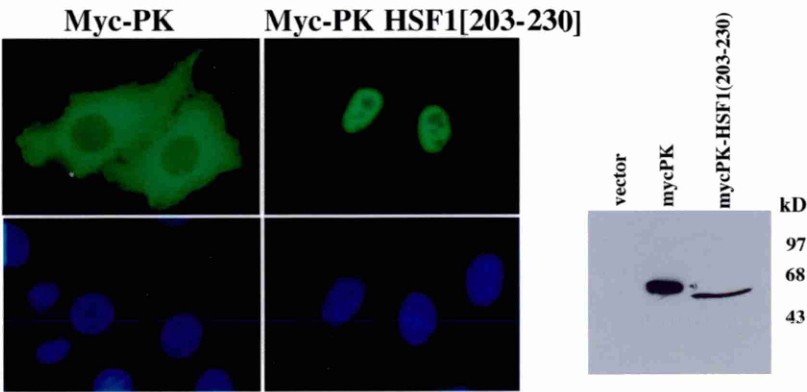
The bipartite NLS of HSF1

A



The NLS of HSF1 promotes nuclear import of pyruvate kinase

B



**Figure 11. Characterization of the bipartite NLS of HSF1.**

- (A) Mutational analysis of the NLS. Schematic representation of mouse HSF1 with the position and the amino acid sequence of the NLS (residues 203-230). The critical residues of the NLS (basic cluster 206-KRK-208, K224 and R227) mutagenized to alanines are indicated by asterisks. Another basic cluster, 116-KRK-118, from the DNA-binding domain (previously indicated by Zuo *et al.*, 1993, as the NLS in human HSF1) was also mutagenized to alanines. The subcellular localization of indicated mutants was assayed by indirect immunofluorescence with anti-HSF1 antibody and typical examples are shown along with corresponding histograms. The subcellular distribution was categorized according to the criteria described in Figure 10. Shown on the right is the immunoblot on whole cell extracts. Endogenous HSF1 is below detection in the lane labelled "vector".
- (B) The bipartite NLS of HSF1 targets myc-tagged chicken pyruvate kinase (myc-PK) to the nucleus of transiently transfected HeLa cells. Immunoblot shown on the right displays expressed species. Note that myc-PK-HSF1(203-230) shows lower molecular weight with respect to myc-PK due to the deletion of PK codons 444-531 by in-frame insertion of the HSF1 NLS.

combined NLS mutations on HSF1 subcellular localization (work in progress). The amino-terminal boundary of the NLS was initially arbitrarily placed at the residue 198 and it was found that the region of HSF1 encompassing residues 198-230 was able to guide a reporter cytoplasmic protein, myc-tagged chicken pyruvate kinase (mycPK) (Michael *et al.*, 1995) to the nucleus (data not shown). This further demonstrated that this region of HSF1 is a NLS, since it works when fused to a heterologous protein. Further deletion up to residue 203 did not influence nuclear residency of mycPK-HSF1(203-230) as shown in Figure 11B. Protein blot shown in Figure 11B confirms that both proteins have the predicted molecular mass, which is reduced for mycPK-HSF1(198-230) in comparison with mycPK, since in-frame fusion of HSF1 fragment (203-230 with a stop codon) to a *KpnI* site in the carboxy-terminus of PK interrupts the reading frame of PK by removing the codons 444-531.

In conclusion, HSF1 contains an NLS that can be defined as bipartite since it comprises two modules consisting of basic amino acids. However, it displays considerable deviation of the sequence from the consensus of the classical bipartite NLS. First, 15, instead of 10 residues separate two basic modules. Secondly, the second module contains two basic residues that are separated by a two amino acid insertion. Third, mutational analysis suggests that the relative contribution of the two modules for nuclear targeting differs, which does not seem to be the case for the classical bipartite NLS, where these modules function interdependently. A similar bipartite NLS

with non-canonical organization was described previously in the human aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor 1 $\alpha$  protein (Eguchi *et al.*, 1997). This NLS also contains two basic modules separated by 13 residues. The first module contains three clustered basic residues (lysine, arginine and arginine), whereas the second, like in the case of HSF1, contains two non-clustered basic residues (lysine spaced by two residues from arginine). By mutational analysis it was found that both basic residues from the second module contribute to nuclear targeting. Interestingly, this NLS was found to drive nuclear import mediated by importins  $\alpha$  and  $\beta$  in *in vitro* assay with permeabilized cells. It would be of interest to see whether the NLS of HSF1 also utilizes this import pathway.

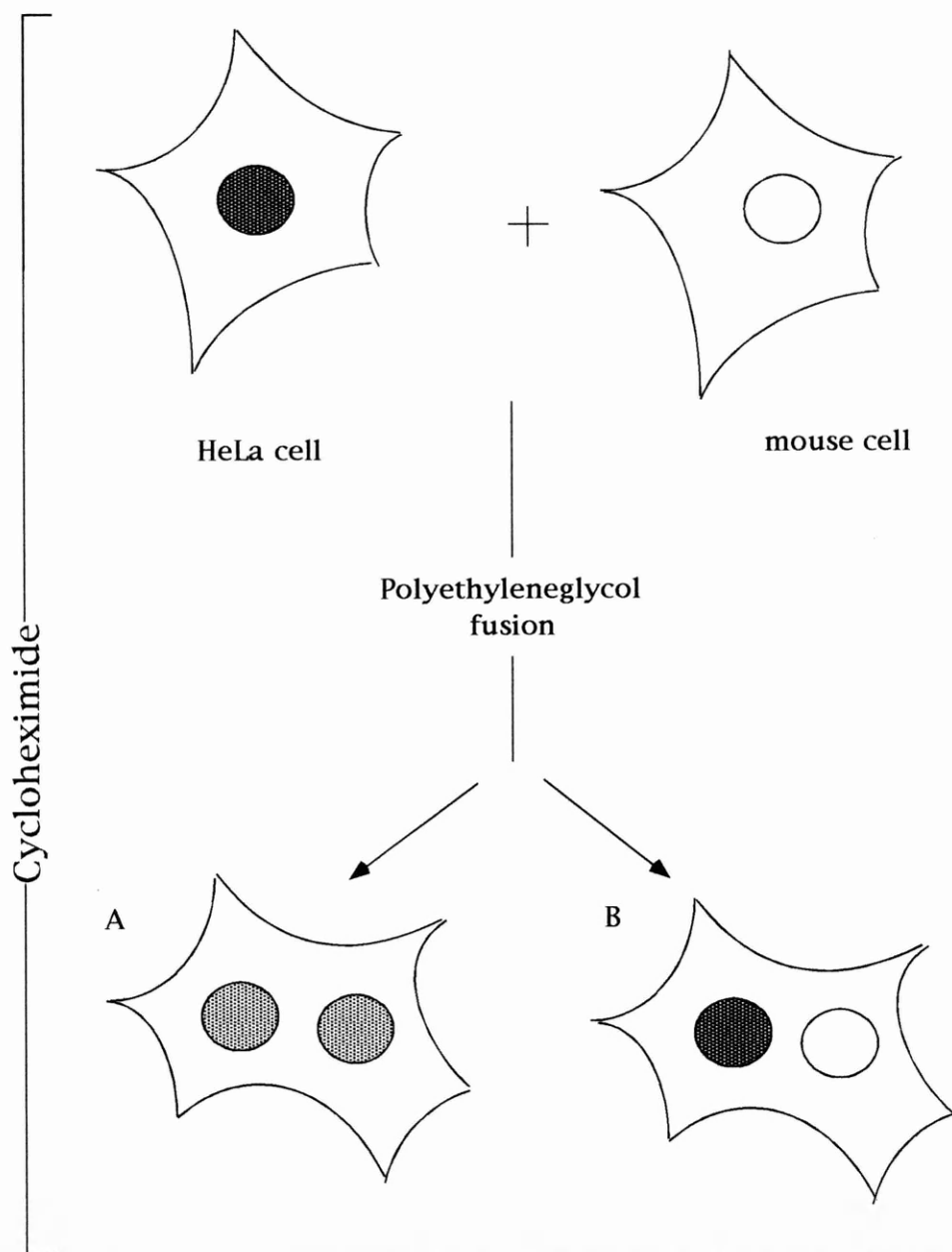
As mentioned above, previous work has identified a basic cluster at position 116 to 118 in human HSF1 to be NLS of HSF1 (Zuo *et al.*, 1995). Using cell fractionation, these authors found that, HSF1 in which these residues were mutagenized to alanines remained in the cytoplasm after heat shock. The same group also found that the mutant HSF1 missing the basic cluster at the position 206-208 was transcriptionally active on a reporter gene in transfected cells. Therefore, they concluded that the basic cluster 206-208 does not play a role in directing HSF1 to the nucleus. The discrepancy between the results presented in this work and the ones obtained by Zuo and coworkers, could be attributed to the difference in the assays used, since subcellular fractionation, as discussed above, may not reflect the accurate distribution of the



protein *in vivo*. Moreover, the effect of amino acid substitutions of the basic cluster at position 206-208, although it does not reveal the contribution of each residue in nuclear targeting, confirms the effect on protein localization in the context of the full length HSF1. Since the region of HSF1 (203-230) containing these residues drives the import of the heterologous protein myc-PK to the nucleus, this clearly shows that this sequence is the major NLS in HSF1. Interestingly, a constitutive NLS of *Drosophila* HSF has been shown to be bipartite containing two basic clusters separated by a short linker (Orosz *et al.*, 1996). This NLS resides in the internal region between the trimerization domain and the carboxy-terminal leucine zipper. In addition, two bipartite NLSs have been found in HSF2, a non-heat shock responsive member of HSF family in mammals (Sheldon *et al.*, 1993; Erratum, 1994).

## HSF1 is a shuttling protein

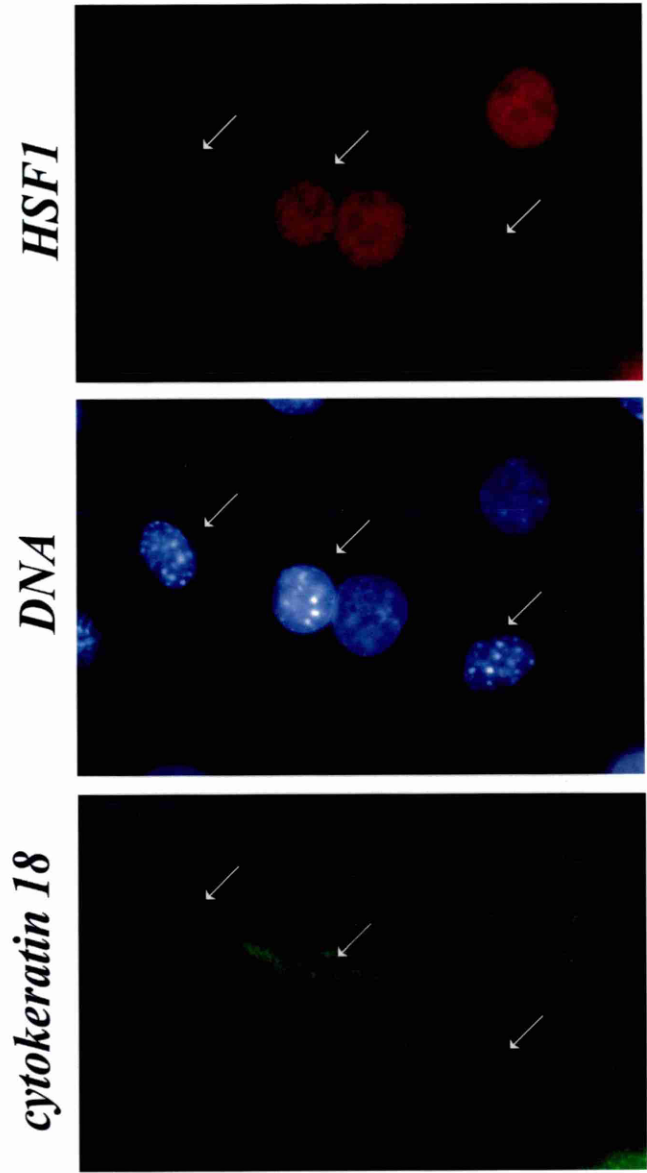
In the experiments described above, HSF1 has been shown to have a potential for bidirectional movement (nucleocytoplasmic shuttling). However, the experimental approach used to reveal this behavior in intact cell (treatment with energy poisons), although not causing irreversible damage, was impairing normal cell metabolism. To show nucleocytoplasmic shuttling by an independent method transient interspecies heterokaryons were used (Figure 12). This method involves the formation of hybrids by polyethylene glycol (PEG) fusion of cells expressing a certain nuclear protein with other cells devoid of this protein. The transfer of the protein from one nucleus to another, in conditions where *de novo* protein synthesis is blocked, demonstrates the transit of the protein through the cytoplasm (Borer *et al.*, 1989; Powell and Burke, 1990; Guichon-Mantel *et al.*, 1991). This assay has been widely used in the field of nucleocytoplasmic transport for different kinds of proteins including nucleolar proteins (Borer *et al.*, 1989), transcription factors (Gauchon-Mantel *et al.*, 1991; Stommel *et al.*, 1998), RNA-binding proteins (Michael *et al.*, 1995; Michael *et al.*, 1997; Càceres *et al.*, 1997) or viral regulatory proteins (Meyer and Malim, 1994; Dobbelstein *et al.*, 1997). Thus, HeLa cells expressing endogenous HSF1 were fused with mouse embryonic fibroblasts (HSF1<sup>-/-</sup>) derived from HSF1 null mice (McMillan *et al.*, 1998) and therefore devoid of HSF1. Treatment with cycloheximide prevented neo-synthesis of



**Figure 12.** To assay nucleocytoplasmic shuttling ability of a protein of interest, HeLa cells expressing the protein were fused with mouse fibroblasts devoided of the protein by treatment with polyethylene glycol. After the incubation at 37°C for a certain time in the presence of the protein synthesis inhibitor cycloheximide (100µg/ml), the heterokaryons were fixed and subjected to immunofluorescence. Two possible outcomes of such an experiment are shown: (A) The appearance of the protein in the mouse nucleus indicates internuclear transfer, demonstrating that the protein is the shuttling protein. The shuttling protein eventually equilibrates between the nuclei of the heterokaryon (shown as an equal gray intensity). (B) In contrast, if no signal can be detected in the mouse nuclei, the protein is confined to the nucleus of HeLa cell. The heterokaryons were delineated by staining of the cytoplasm with human-specific monoclonal anti-cytokeratin 18 antibody, anti-β galactosidase antibody in case when mouse cells expressing cytoplasmic β galactosidase were used or by staining of actin with phalloidin. Mouse nuclei were distinguished by specific dotted pattern of DNA staining with fluorescent dye Hoechst 33258.

HSF1 and 4 hours post-fusion HSF1 distribution was analyzed by immunocytochemistry. Mouse nuclei were identified by fluorescent staining of their satellite DNA with Hoechst 33258 (Borer *et al.*, 1989), while the staining of the cytoplasm with a human-specific anti-cytokeratin 18 monoclonal antibody was used to delineate fused cells. To rigorously investigate internuclear migration, immunostaining was performed with three different anti-HSF1 antibodies, all mentioned above: anti-whole molecule, anti-C term end peptide and Mab (epitope: 378-407). As shown in Figure 13, HSF1 staining intensity was similar in mouse and human nuclei 4 hours post-fusion. Non-fused mouse cells were not stained above background. Internuclear transfer was detected in virtually all heterokaryons inspected, being barely detectable 1 hour post fusion and reaching apparent internuclear equilibration in ~50% of heterokaryons 4 hours post-fusion. The results were essentially the same for heterokaryons stained with the other two anti-HSF1 antibodies, anti C-term end or Mab (data not shown). This experiment yielded the conclusion that HSF1 is not confined within the nucleus but rather shuttles continuously between the nucleus and the cytoplasm, implying a dynamic behaviour of HSF1 in the cell: the protein constantly moves between two compartments, but is at steady state accumulated in the nucleus. Dynamic behavior of proteins that are highly compartmentalized (nuclear) at steady state, is not a unique feature of HSF1. In respect to the class of proteins HSF1 belongs to (transcription factors), this feature was, using heterokaryon assays, described for two

Figure 13



**Figure 13. Nucleocytoplasmic shuttling of endogenous HSF1 in transient interspecies heterokaryons.**

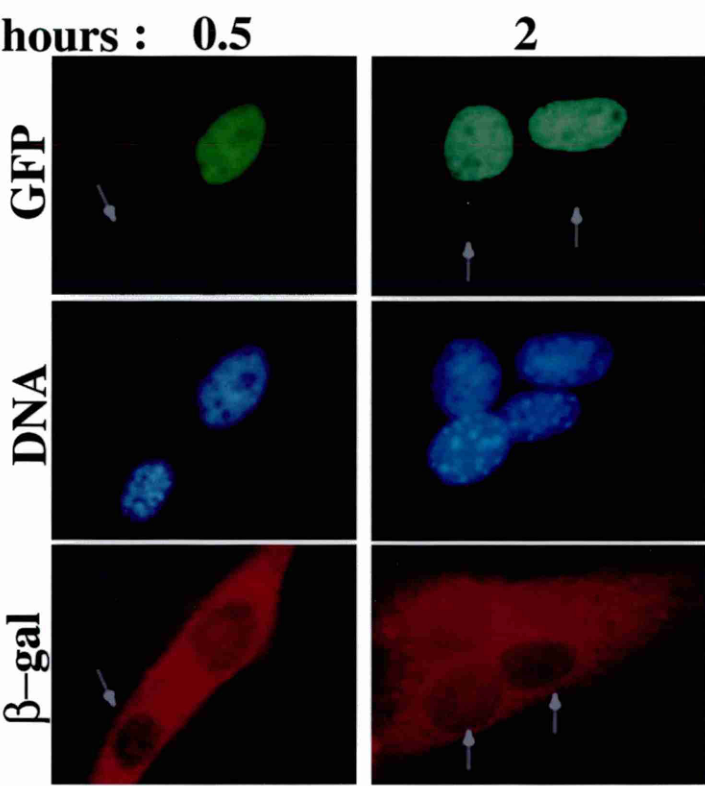
Heterokaryons were formed between HeLa cells and HSF1 null fibroblasts and 4 hours post-fusion stained with anti-HSF1 polyclonal antibody (anti-whole molecule). The arrows point to the mouse nuclei.

other nuclear proteins: progesterone receptor (Gauchon-Mantel *et al.*, 1991) and p53 (Middler *et al.*, 1997; Stommel *et al.*, 1999). Nucleocytoplasmic shuttling was also demonstrated for the glucocorticoid receptor (Maden and DeFranco, 1993), although this protein, when unliganded, is usually localized in the cytoplasm (Picard and Yamamoto, 1987). While in the case of progesterone and glucocorticoid receptors, the functional significance of this phenomenon remains obscure, it has been hypothesized that nucleocytoplasmic shuttling of p53 is essential for its turnover (Roth *et al.*, 1998). Considering HSF1, nucleocytoplasmic shuttling described in this work is a novel property of this transcription factor, known to regulate stress-induced expression of cytoplasmic and nuclear chaperones in mammalian cells. The continuous cycling between these two compartments at physiological temperature suggests an additional level at which this protein operates in the cell and several possible implications for its role. These possibilities are discussed in detail below.

In the experiments presented above, HSF1 was shown to shuttle continuously between the nucleus and the cytoplasm at 37°C. One necessary control experiment was to check whether shuttling is induced by the experimental protocol used i.e. PEG or cycloheximide treatment. For this, nuclear transcription factor c/EPB- $\alpha$ , a member of the mammalian CCAAT/Enhancer-binding protein family (Lekstrom-Himes and Xanthopoulos, 1998), was analyzed in the heterokaryon assay. HeLa cells overexpressing transiently c/EBP- $\alpha$ -GFP were fused with mouse 3T3 derivatives ("G2b2") stably expressing  $\beta$ -

Figure 14

**c/EBP<sub>α</sub>-GFP**





**Figure 14. c/EBP $\alpha$ -GFP is confined to the nucleus.**

Heterokaryons between HeLa cells transiently overexpressing c/EBP $\alpha$ -GFP and "G2b2" mouse fibroblasts were analyzed 30 min or 2 hours post-fusion. The arrows point to the mouse nuclei.

galactosidase in the cytoplasm. Unlike HSF1, c/EBP- $\alpha$  tested negative for internuclear transfer in most of the heterokaryons observed 30 min or 2 hours post fusion (Figure 14). In a few heterokaryons a signal in mouse nuclei was barely above the background level and did not seem to increase over time. From this experiment it can be concluded that experimental conditions or protein overexpression *per se* do not induce shuttling.

Heterokaryon experiments reveal a dynamic behavior of HSF1 in apparent absence of stress stimuli, when HSF1 is maintained in an inactive, monomeric state. This dynamic behaviour is also observed for a GFP-HSF1 chimera, whose state was properly maintained upon transient expression in HeLa cells (Figure 9C). GFP-HSF1 showed internuclear transfer in heterokaryon assays with kinetics similar to the endogenous protein, reaching apparent internuclear equilibration 4 hours post-fusion (Figure 15A). This situation was observed in ~50% of heterokaryons that scored positive for shuttling (89%). Unlike endogenous protein, overexpressed HSF1 was clearly detected in mouse nuclei at earlier time points, 2 hours (Figure 15A) or 30 min post-fusion (Figure 15B). Two heterokaryons illustrate the range of signal observed in recipient nuclei of most of the heterokaryons inspected for 30 min time point. Therefore, GFP-HSF1 allows an analysis of internuclear transfer at early time points, which is not practical with endogenous HSF1 due to its low abundance. The use of GFP-HSF1 also makes it possible to examine shuttling during heat stress without

Figure 15A

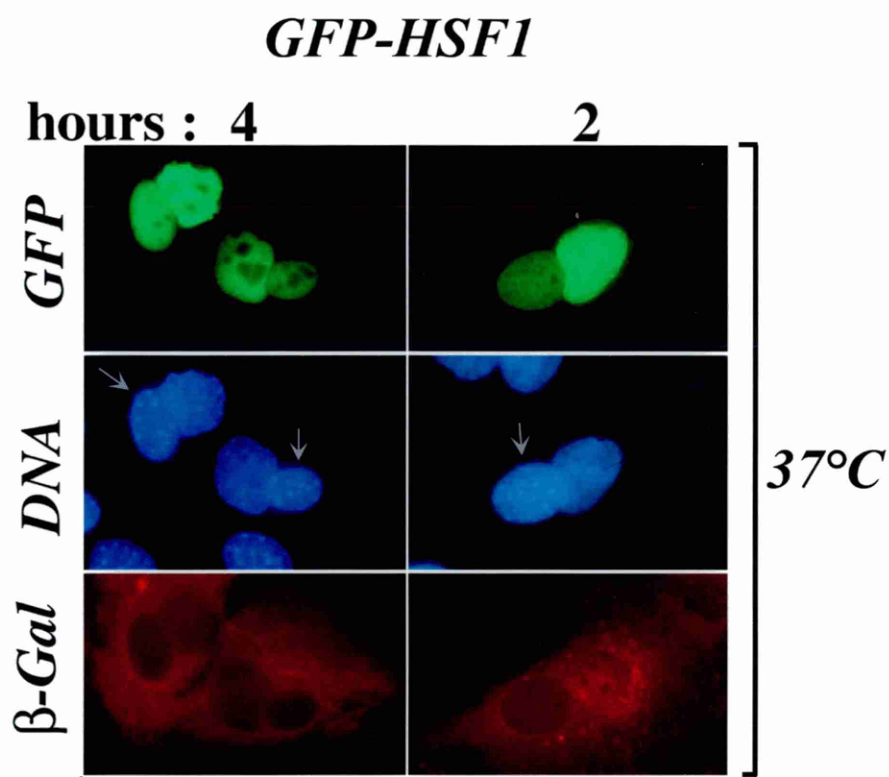
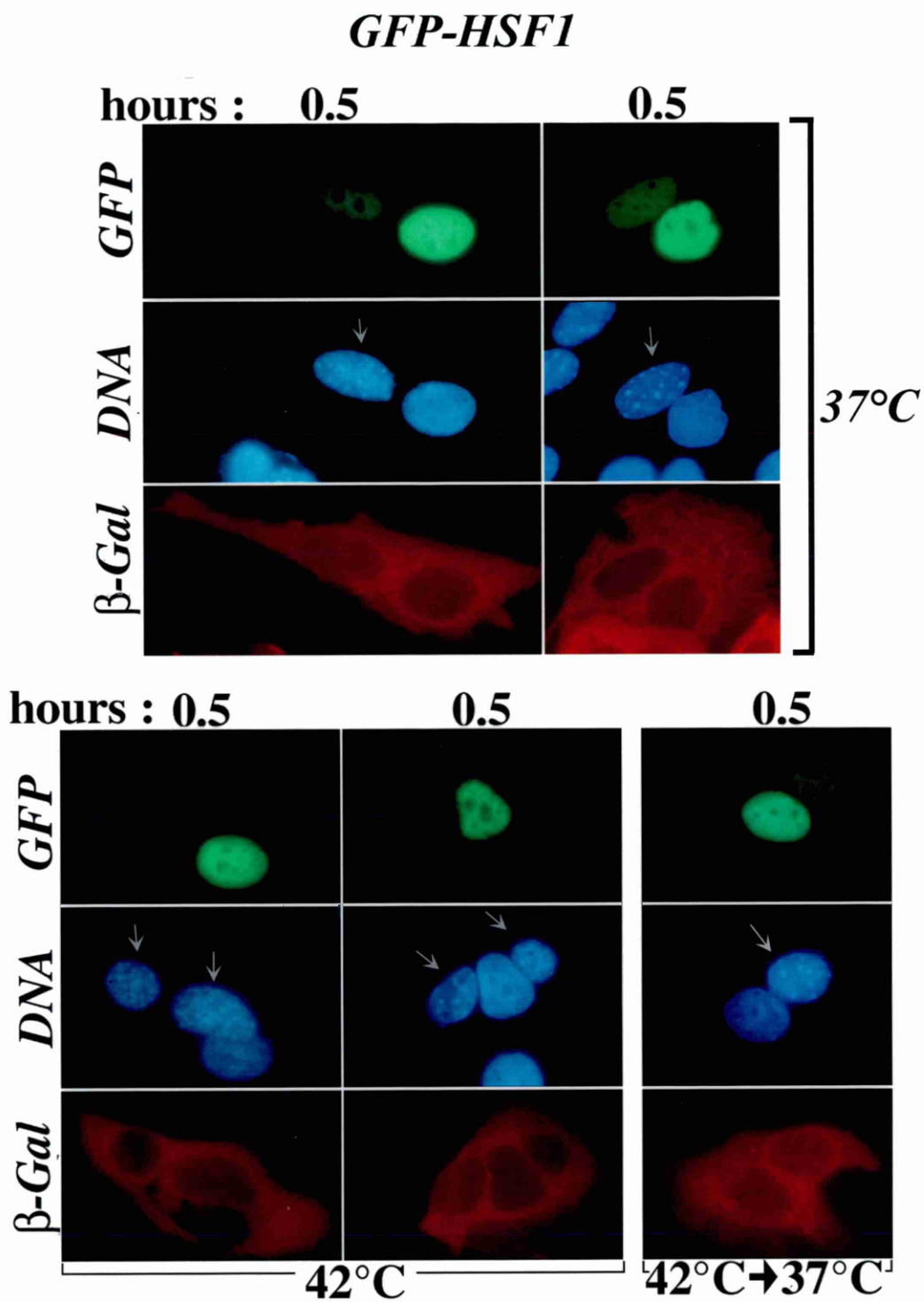


Figure 15B



**Figure 15. Nucleocytoplasmic shuttling of GFP-HSF1 is regulated by heat shock.**

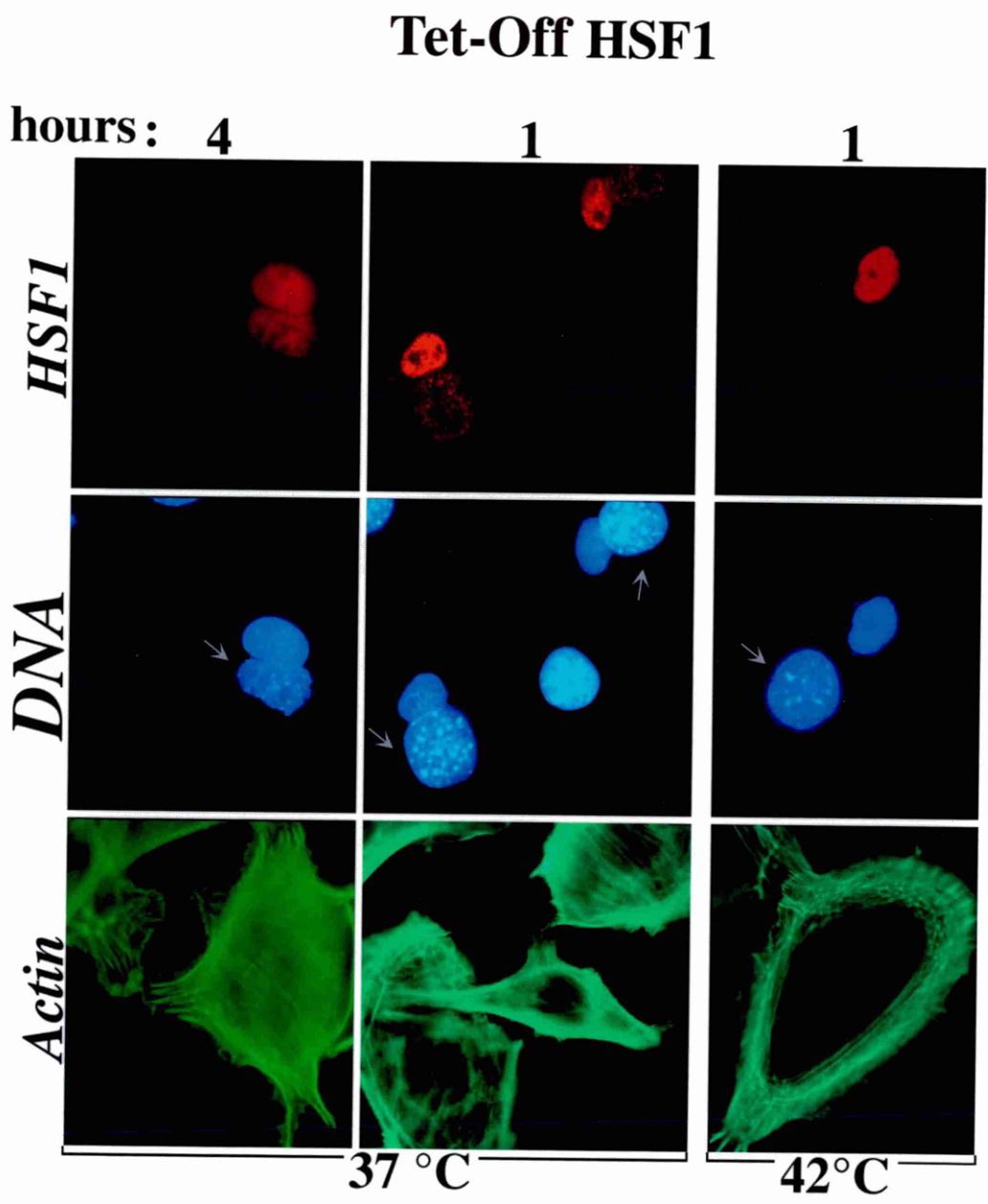
(A) Nucleocytoplasmic shuttling of GFP-HSF1 chimera detected 4 hours or 2 hours post-fusion.

(B) Heat shock reversibly blocks nucleocytoplasmic shuttling of GFP-HSF1.

After cell fusion, the heterokaryons are incubated at 37°C for 30 min, or heat shocked at 42°C for 30 min, or heat shocked and then recovered at 37°C for 30 min. Two heterokaryons shown for 30 min time point represent typical examples for a range of internuclear transfer observed. The arrows point to the mouse nuclei.

prolonged incubations. For this, heterokaryons were immediately after fusion subjected to 42°C for 30 min, followed by fixation and immunostaining. As shown in Figure 15B, internuclear transfer of GFP-HSF1 is impaired, since no signal can be detected in mouse nuclei. This situation was seen in 93% of heterokaryons inspected. This experiment was also done in a slightly different way, with HeLa/G2b2 cocultures being heat shocked at 42°C for 30 min prior to fusion, after which the heat shock was continued for an additional 30 min, giving identical results (data not shown). This block of internuclear transfer is however reversible: if heat shocked heterokaryons are shifted back to 37°C for 30 min shuttling is detected in the majority of heterokaryons (60%), as shown by a representative immunostaining in Figure 15B. Thus, the block of nucleocytoplasmic shuttling of GFP-HSF1 seen during the heat shock correlates with the monomer to oligomer transition (shown in Figure 9C). In addition, shuttling resumed during recovery when GFP-HSF1 dissociates to its inactive form (Figure 9C). To exclude the possibility that the absence of GFP-HSF1 signal in mouse nuclei is due to the attenuation of the GFP chromophore during the heat shock, a control experiment was performed using clone 36 cells, in which non-tagged HSF1 ("Tet-Off"HSF1) is also properly regulated (Figure 7C). At 37°C, HSF1 was detected in nuclei of HSF1-/- fibroblasts 60 min post-fusion in most heterokaryons (94%); after 4 hours the signal reached an apparent equilibrium distribution in ~50% (Figure 16). Therefore, "Tet-Off" HSF1 behaves like endogenous HSF1 and

Figure 16



**Figure 16. Heat shock regulates nucleocytoplasmic shuttling of stably expressed HSF1.**

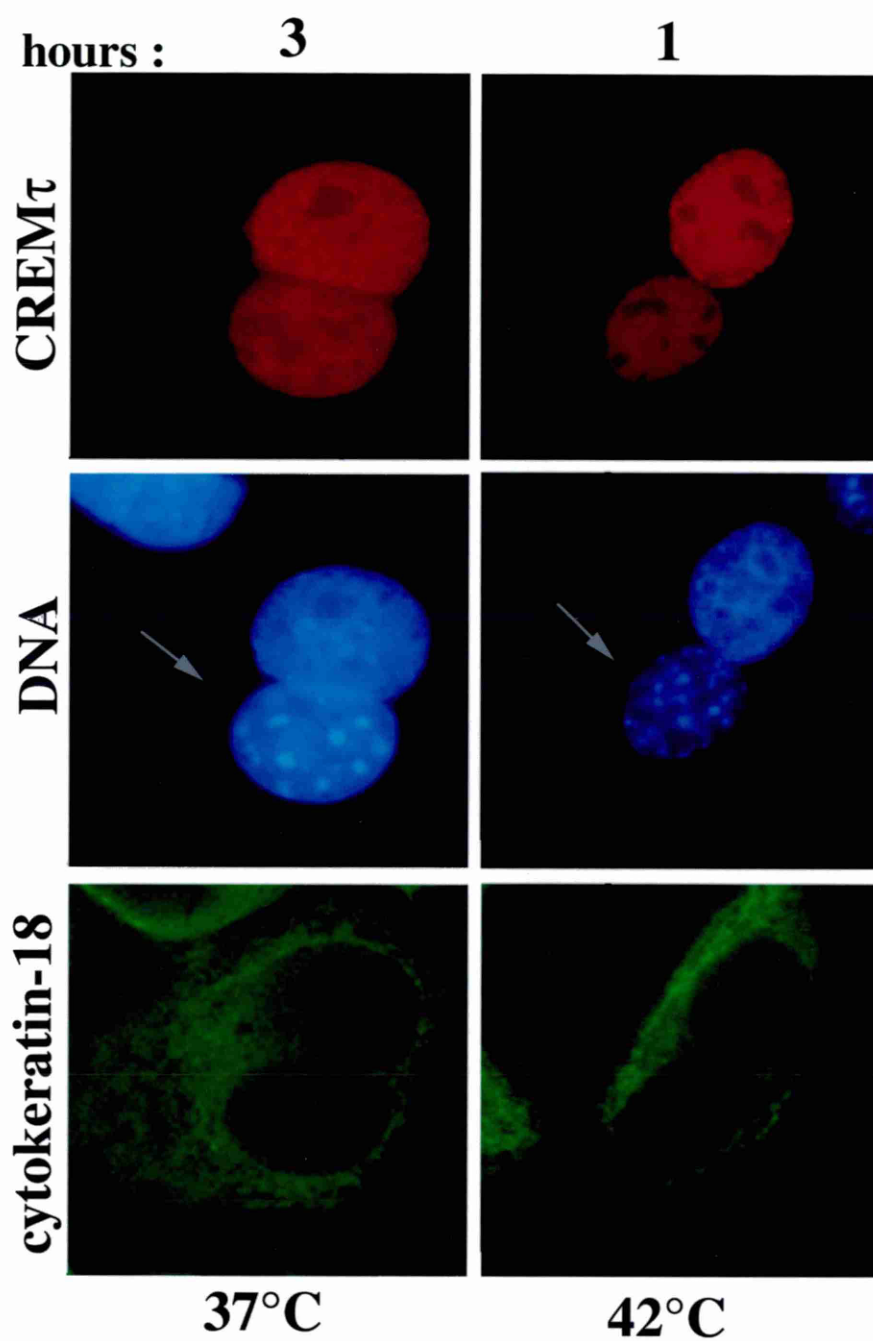
Heterokaryons between clone 36 cells and HSF1 null fibroblasts were maintained at 37°C for 1 hour or 4 hours, or heat shocked at 42°C for 1 hour. The arrows point to the mouse nuclei.



transiently expressed GFP-HSF1, with the similar kinetics of internuclear transfer. In heterokaryons subjected to heat shock at 42°C for 60 min, internuclear transfer of "Tet-Off" HSF1 was blocked in 85% of cases, as shown in a representative example in Figure 16. In addition, since heat shock in the case of clone 36 cells was performed for 60 min, this makes it unlikely that the absence of internuclear transfer is due to a delay of shuttling, at least within the time frame examined (30 and 60 min). Clearly, under the conditions of heat stress when HSF1 is converted to its active form, nucleocytoplasmic shuttling is blocked. This block is, however, reversible since shuttling restores during recovery.

Heat shock has been reported previously to block the export of mRNAs from the nucleus in yeast, with the exception of mRNAs for certain heat shock proteins (Saavedra *et al.*, 1996; Saavedra *et al.*, 1997). However, there are no data concerning the effect of heat shock on nucleocytoplasmic shuttling of proteins, neither in yeast nor mammals. Interestingly, heat shock seems to specifically regulate nucleocytoplasmic shuttling of HSF1, but not of another transcription factor unrelated to HSF1. CREM $\tau$  is a member of the CREM family of mammalian transcription factors which mediates transcriptional response induced by elevated levels of cAMP (Sassone-Corsi, 1995). Both HeLa and G2b2 cells stained with anti-CREM antibody showed an undetectable level of endogenous protein (data not shown). This allowed us to trace the behaviour of overexpressed CREM $\tau$  in the heterokaryon assay. Like HSF1, CREM $\tau$  showed shuttling in the

Figure 17



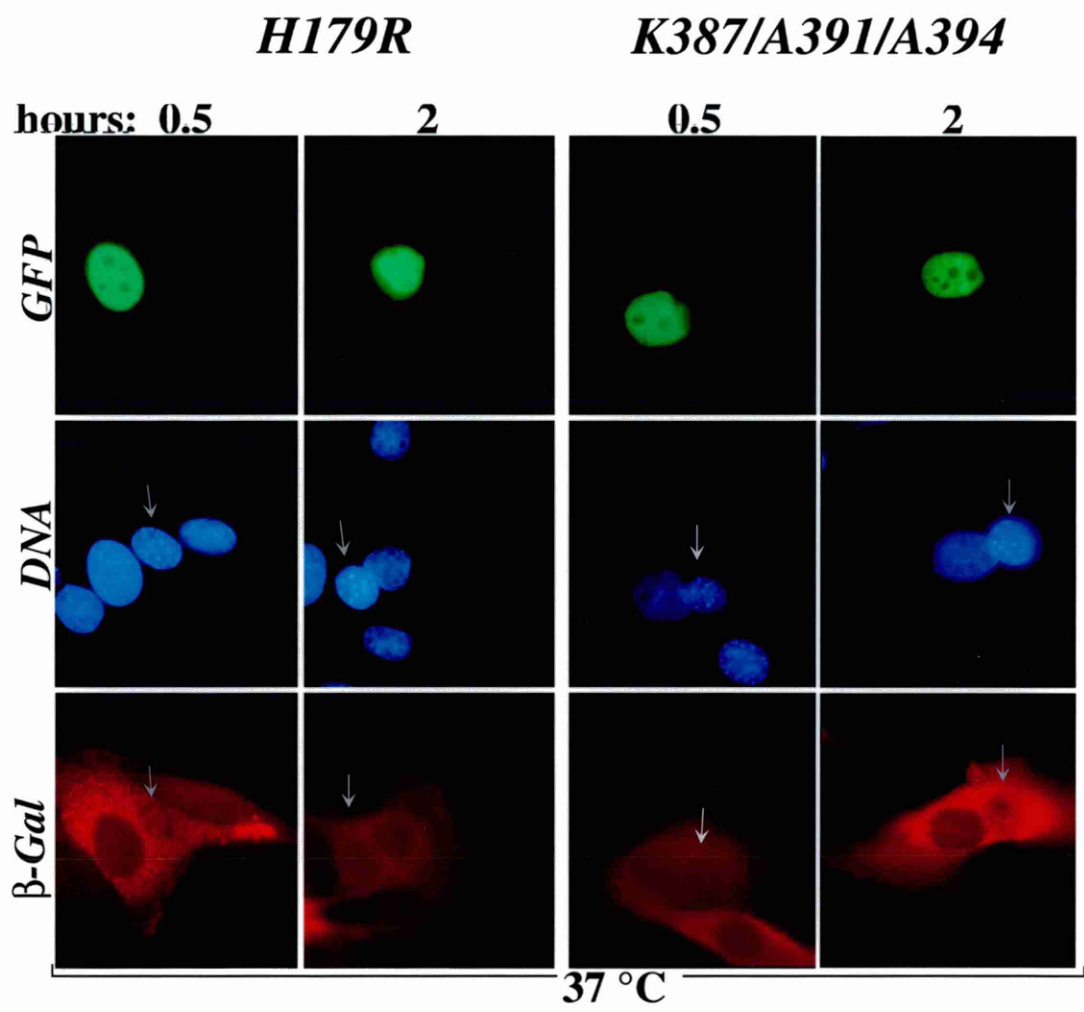
**Figure 17. Nucleocytoplasmic shuttling of CREM $\tau$  is refractory to heat shock.**

Heterokaryons between HeLa cells transiently overexpressing CREM $\tau$  and "G2B2" fibroblasts were incubated at either 37°C for 3 hours or at 42°C for 1 hour. CREM $\tau$  was detected with polyclonal anti-CREM antibody. The arrows point to the mouse nuclei.

majority of the heterokaryons inspected, the signal reaching an apparent internuclear equilibrium 3 hours post fusion (Figure 17). In contrast to HSF1, internuclear transfer was maintained in the majority of heterokaryons incubated at 42°C for 1 hour (Figure 17). This result suggests that nucleocytoplasmic shuttling competence is still functional under the conditions of mild heat shock used. Therefore, the block of HSF1 shuttling at 42°C may not be due to the general inhibition of transport competence. Rather, it seems to be specific for active, trimeric HSF1. This could be due either to binding to chromatin, or to the fact that the sequence(s) directing the export of HSF1 are buried in the oligomer, or both. Clearly, to distinguish between these possibilities, further experiments are necessary (see Discussion).

To gain further insight into the relation between the oligomeric state of HSF1 and its shuttling ability, two mutations that make HSF1 constitutively oligomeric were tested in the heterokaryon assay. As described above (Figure 9C) these mutations consist of a single amino acid substitution, H179R, or triple substitution M387K/L391A/L394A, which derepress oligomerization. In this way, the oligomeric state of HSF1 can be altered at physiological temperature thus mimicking heat-induced oligomerization. Interestingly, both mutations impair shuttling at 37°C (Figure 18). In the case of the H179R mutant, internuclear transfer was blocked in 89% of the heterokaryons inspected 30 min post-fusion, whereas 2 hours post-fusion 68% of heterokaryons scored negative for

Figure 18



**Figure 18. Nucleocytoplasmic shuttling is impaired for constitutively oligomeric HSF1.**

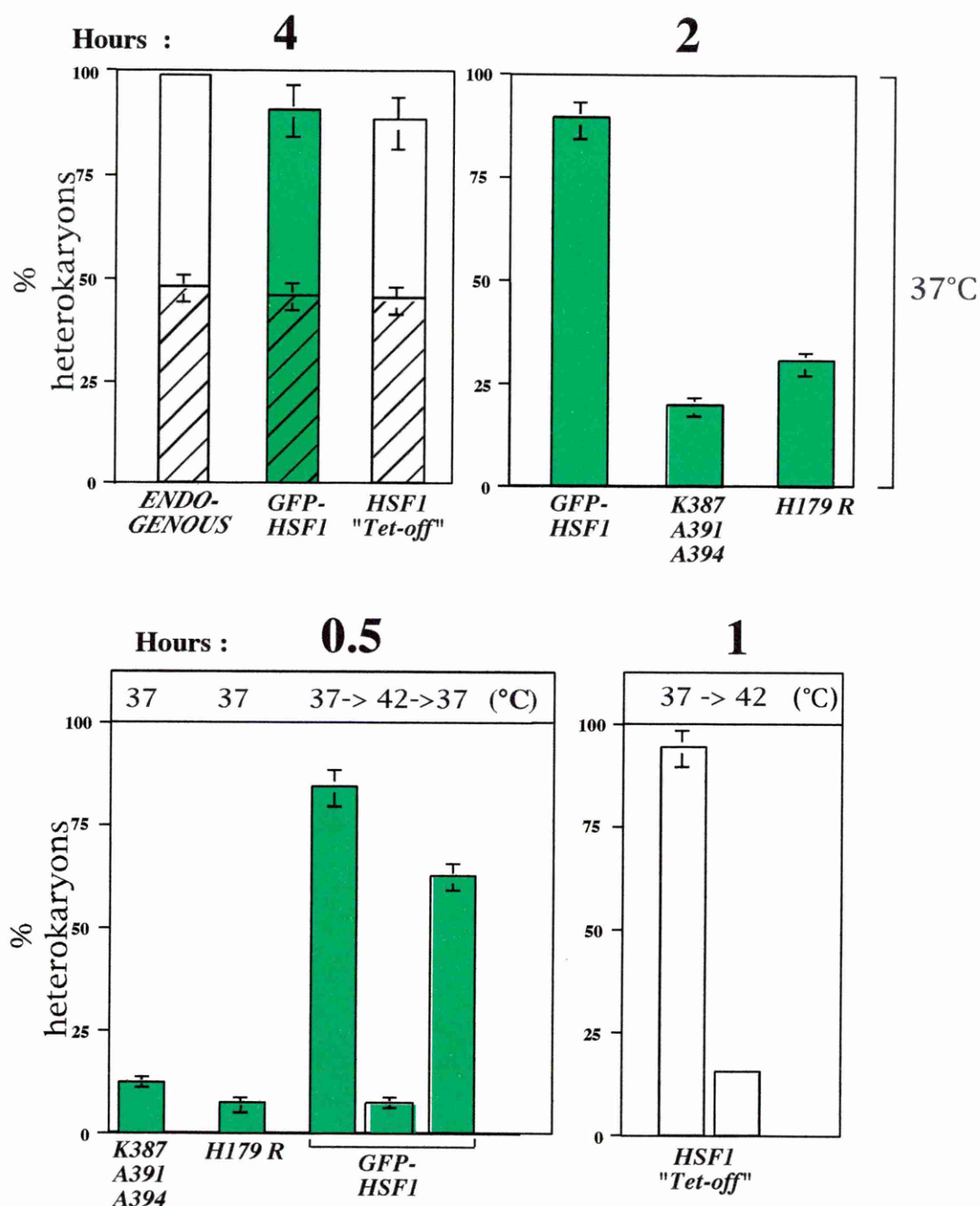
Heterokaryons between HeLa cells transiently overexpressing oligomeric HSF1 mutants (GFP-HSF1: H179R or K387/A391/A394) and "G2b2" fibroblasts were maintained at 37°C for 30 min or 2 hours. The arrows point to the mouse nuclei.

internuclear transfer. Similar behavior was observed for M387K/L391A/L395A. In this case, shuttling was blocked in 88% and 81% of heterokaryons 30 min or 2 hours post-fusion respectively (Figure 18). For both mutants, a fraction of heterokaryons scored weakly positive for internuclear transfer presumably due to shuttling of a residual monomeric fraction. This behaviour was reproducibly seen in several experiments. Since both heat shock and mutations that convert HSF1 to its active, oligomeric form impaired shuttling, this strongly suggests that it is the inactive, monomeric form of HSF1 to be subjected to continuous movement between compartments. Why does oligomeric HSF1 not shuttle? As mentioned above, HSF1 oligomer possesses high affinity for binding to HSEs in chromatin and could be retained in the nucleus during heat shock. Alternatively, oligomers are not accessible to the export machinery due to a conformational change, which hides signals required for *nuclear export activity*. These two possibilities are not mutually exclusive. Constitutively oligomeric mutants could behave in the same way being retained in the nucleus at 37°C. Alternatively, the substitutions that derepress oligomerization may inactivate overlapping sequence elements required for nuclear export activity, which are positioned in two physically separated protein domains (trimerization domain and leucine zipper 4). From the mechanistic point of view, it can be inferred from the heterokaryon assay that the block of shuttling of HSF1 observed during heat shock, or of oligomeric mutants at normal growth temperature, is probably due to the block of

export. In both cases HSF1 neither appeared in the mouse nuclei, neither did it was accumulated in the cytoplasm of the heterokaryons.

The outline of the shuttling behaviour of HSF1, endogenous or overexpressed is shown in Figure 19. The histograms summarize results of many experiments in which HSF1 was tested for internuclear transfer in transient heterokaryons. For each form of HSF1, >100 heterokaryons were scored by visual inspection after immunofluorescence staining. During the course of this work, an interesting behaviour of the GFP-HSF1: $\Delta$ 160-172 mutant was detected in the heterokaryon assay. This mutant could be used to find out whether the block of shuttling at 42°C occurs even when HSF1 is unable to oligomerize. Instead, this mutation caused a considerable delay of internuclear transfer at 37°C. The signal in the recipient nucleus 2 or 4 hours post-fusion was roughly comparable with the signal observed in the majority of heterokaryons with GFP-HSF1wt, 30 min post-fusion (Figure 20). This suggests that residues 160-172 may directly or indirectly participate in the nuclear export activity of HSF1. It is intriguing that this mutation falls in the same region of HSF1 (trimerization domain) as the other mutation described above, H179R, which impaired the shuttling ability of HSF1 more severely, but also has the opposite phenotype with respect to the  $\Delta$ 160-172 mutant, in that it makes HSF1 oligomeric. Whether or not this is due to the presence of the sequence responsible for nuclear export activity of HSF1 in the trimerization domain is currently under investigation.

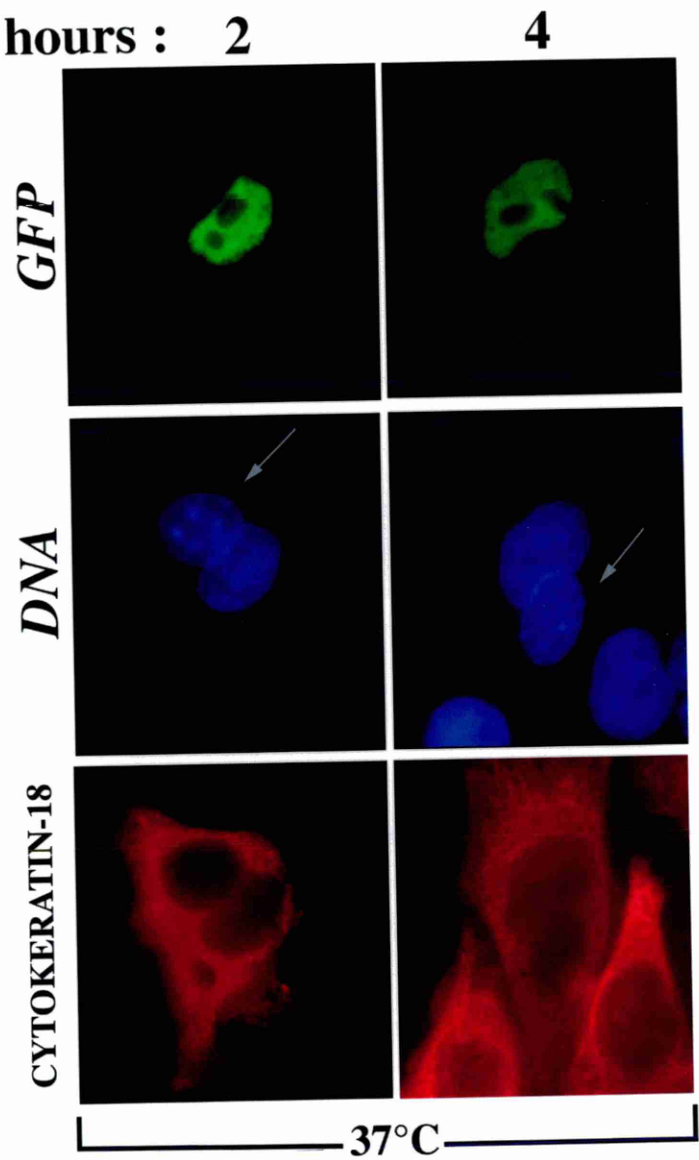




**Figure 19.** Summary of the shuttling behavior of HSF1 in the heterokayon assay. The histograms represent results of scoring >100 heterokaryons of either endogenous and stably expressed HSF1 (shown in gray) or transiently expressed GFP-HSF1 chimeras (shown in green). At 4 hours post-fusion, an apparent internuclear equilibration was observed in 50% of heterokaryons (shown as a dashed area of respective histograms).

Figure 20

$\Delta 160-172$



**Figure 20. Nucleocytoplasmic shuttling is delayed for HSF1 lacking a region (amino acids 160-172) of the trimerization domain.**

Heterokaryons between HeLa cells transiently overexpressing GFP-HSF1:Δ160-172 and "G2b2" fibroblasts were maintained at 37°C for 2 hours or 4 hours. The arrows point to the mouse nuclei.

Arguing in favour of this possibility are the results of carboxy-terminal truncations of HSF1 (Figure 10A).

The experiments presented above yielded several important conclusions: first, HSF1 shuttles continuously between the nucleus and the cytoplasm at physiological temperature; second, heat shock reversibly blocks nucleocytoplasmic shuttling; third, this block can be recapitulated at physiological temperature using HSF1 mutants which are constitutively oligomeric. Taken together, this implies that the inactive, monomeric HSF1 undergoes nucleocytoplasmic trafficking, as shown in a possible model of nucleocytoplasmic trafficking of HSF1 (Figure 21). This model proposes that inactive HSF1 is not statically confined to the nucleus, but rather has a capacity to traverse the NPC. However, at steady state, inactive HSF1 is accumulated in the nucleus, presumably due to the higher rate of import than export (shown as a bold arrow). When HSF1 is converted to its active, trimeric form, nucleocytoplasmic shuttling is impaired. If oligomerization of HSF1 is induced by heat stress, this block is reversible and shuttling resumes during the recovery period when oligomers are being dissociated. The block observed during heat shock or for oligomeric mutants at physiological temperature seems to reflect a property of HSF1 oligomers. Nucleocytoplasmic shuttling of CREM $\tau$  continues during heat stress, indicating that the general transport competence of the cells subjected to mild heat stress is not abrogated. Therefore, HSF1 oligomers are either retained in the nucleus through specific association with

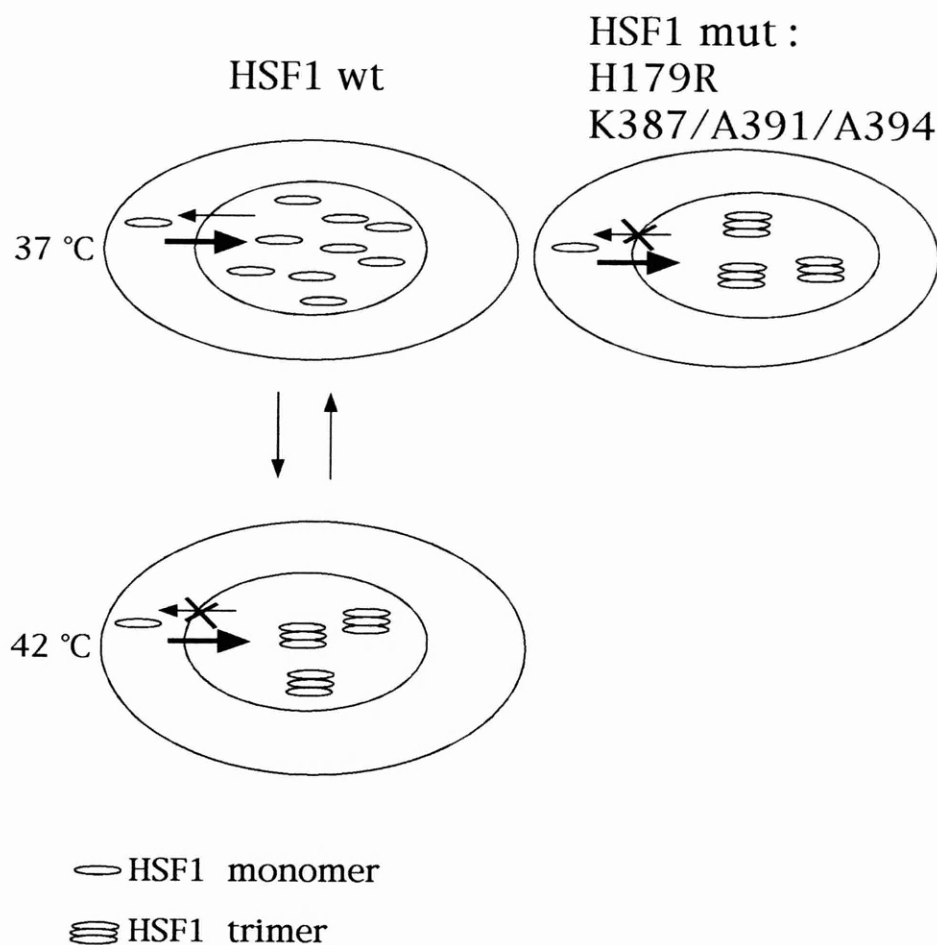


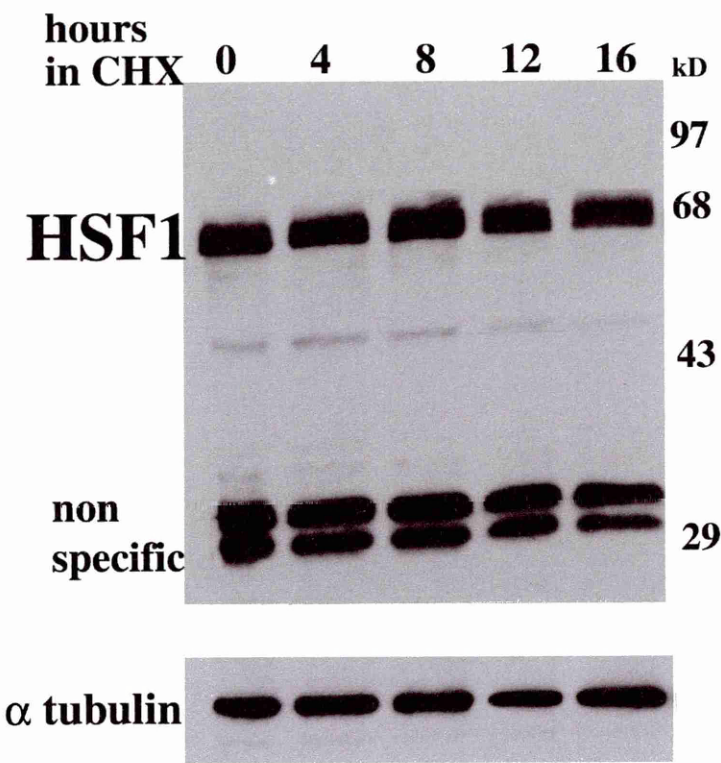
Figure 21. A possible model for nucleocytoplasmic shuttling of HSF1 in mammalian cell. HSF1 shuttles continuously between the nucleus and the cytoplasm, but is, at steady state, accumulated in the nucleus presumably due to the higher import rate (shown as a bold arrow). Shuttling is impaired at 42°C for the wild type HSF1 or at 37°C for the oligomeric mutants presumably due to the block of export of HSF1 oligomers suggesting that only inactive, monomeric HSF1 shuttles.

chromatin or some other nuclear components. Alternatively, oligomers may not access the export machinery. One possible reason would be, for example, a conformational change that would inactivate or bury the nuclear export activity in HSF1. It should also be considered that HSF1 is a phosphoprotein and undergoes hyperphosphorylation on serine and threonine residues upon heat stress (Xia and Voellmy, 1997). Although the exact role of phosphorylation is not known, except its proposed involvement in suppression of transcriptional activity of HSF1 at 37°C (Knauf *et al.*, 1996; Kline and Morimoto, 1997), this modification might also influence the shuttling capacity. Such a situation has been described for the glucocorticoid receptor, where hyperphosphorylation at specific sites is associated with inefficient nucleocytoplasmic shuttling (DeFranco *et al.*, 1991). Since HSF1 contains 61 serines and 29 threonines this makes a systematic analysis of the possible role of hyperphosphorylation in regulation of HSF1 transport cycle very difficult. Therefore, assessment of the putative role of phosphorylation awaits the identification of phosphorylated residues. In addition, it has been recently reported that another post-translational modification, acetylation, can influence shuttling behavior of the liver-specific transcription factor HNF4, causing its nuclear retention (Soutoglou *et al.*, 2000). It is not known whether HSF1 is also subjected to acetylation, but in the light of the emerging role of this modification on transcription factor function, this possibility can not be disregarded.

The model of nucleocytoplasmic trafficking of HSF1 presented above, inevitably raises the question of how this continuous flow of HSF1 between two cellular compartments is related to the stability of HSF1 during the cell cycle. To address this question, clone 36 cells were, upon tetracycline removal, treated with the protein synthesis inhibitor, cycloheximide. As for the heterokaryon assays, at different time points the cells were collected and protein extracts were analyzed by immunoblotting with anti-HSF1 antibody. The results showed that, under these conditions, HSF1 is a stable protein because no apparent change in the amount of HSF1 can be observed within 20 hours of cycloheximide treatment (Figure 22). The same filter was also probed with anti- $\alpha$ -tubulin monoclonal antibody to normalize total protein input.  $\alpha$ -tubulin has a very slow turnover (half-life between one and five days, Bohley, 1996). In agreement with these data, the amount of  $\alpha$ -tubulin remained apparently constant during 20 hours of incubation with cycloheximide (Figure 22). From this experiment it can be concluded that moderately overexpressed HSF1 is a stable protein under conditions where *de novo* protein synthesis is inhibited. However, the half-life of HSF1 also needs to be determined by pulse-chase experiment (work in progress).

It has been demonstrated by quantitative immunoblot analysis that a HeLa cell contains on average 30,000 molecules of HSF1 (Sarge *et al.*, 1993). Since an apparent internuclear equilibration is seen in ~50% of heterokaryons 4 hours after fusion of HeLa cells and HSF1<sup>-/-</sup> mouse fibroblasts, a very

Figure 22





**Figure 22. Analysis of HSF1 stability in clone 36 cells.**

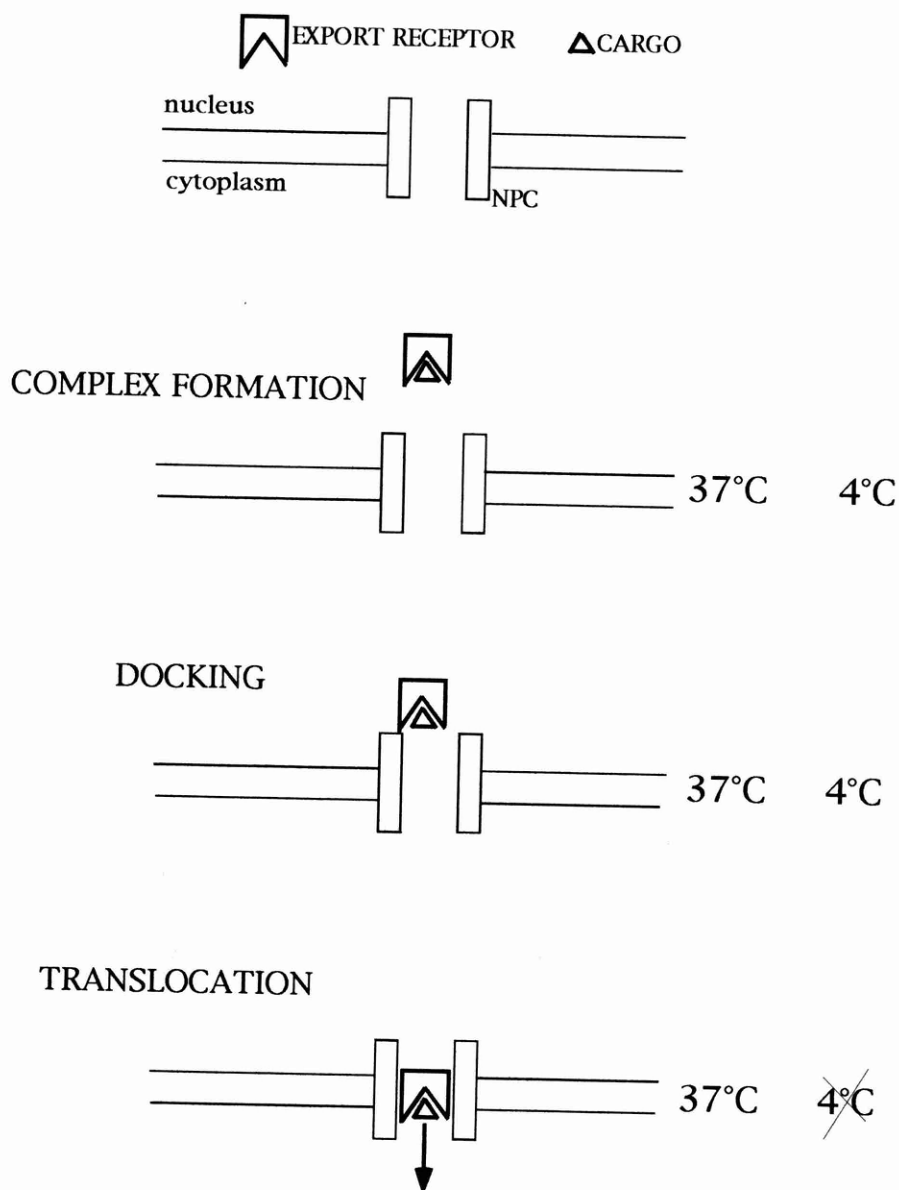
Clone 36 cells were incubated in the medium containing cycloheximide (CHX :100 $\mu$ g/ml) for indicated periods of time and then analyzed by anti-HSF1 immunoblot. The same filter was reprobed with monoclonal anti- $\alpha$  tubulin antibody.

conservative (minimal) estimate gives an export rate of 1 molecule per second. Consequently, during the time in which the internuclear equilibration is reached (4 hours), the whole pool of endogenous protein would have shuttled once. However, this estimate is to be quadrupled if it is taken into account that the heterokaryon system provides a bidirectional transport assay. Average length of the interphase of HeLa cell is ~20 hours (Lodish *et al.*, 2000) and assuming that endogenous HSF1 in HeLa cells is a stable protein, an export rate of 1 molecule/sec implies that each molecule of HSF1 shuttles in and out of the HeLa nucleus several times during interphase. It is interesting that, comparing the export rate of HSF1 with the export rate of some other shuttling proteins such as hnRNPA1 (Michael *et al.*, 1995), that the fraction of the total protein pool that is exported per unit time (0.003%/sec), is roughly the same for both proteins. Unlike HSF1, hnRNPA1 is a much more abundant protein in mammalian cell ( $7 \times 10^7$ - $10 \times 10^7$  molecules/cell). Thus, the export rate for both proteins is fast in comparison with some other shuttling proteins, such as progesterone receptor (Guichon-Mantel, 1991) and nucleolin (Borer *et al.*, 1989).

Import and export of the proteins from the nucleus is considered to be a selective and signal-mediated process, although in principle proteins with a molecular mass up to 40-60 kD can pass through the NPC aqueous channel freely (Nigg, 1997). Ability of a protein to diffuse depends on its functional size in the cell, which can be significantly different from its predicted size (Talcott *et al.*, 1999). The

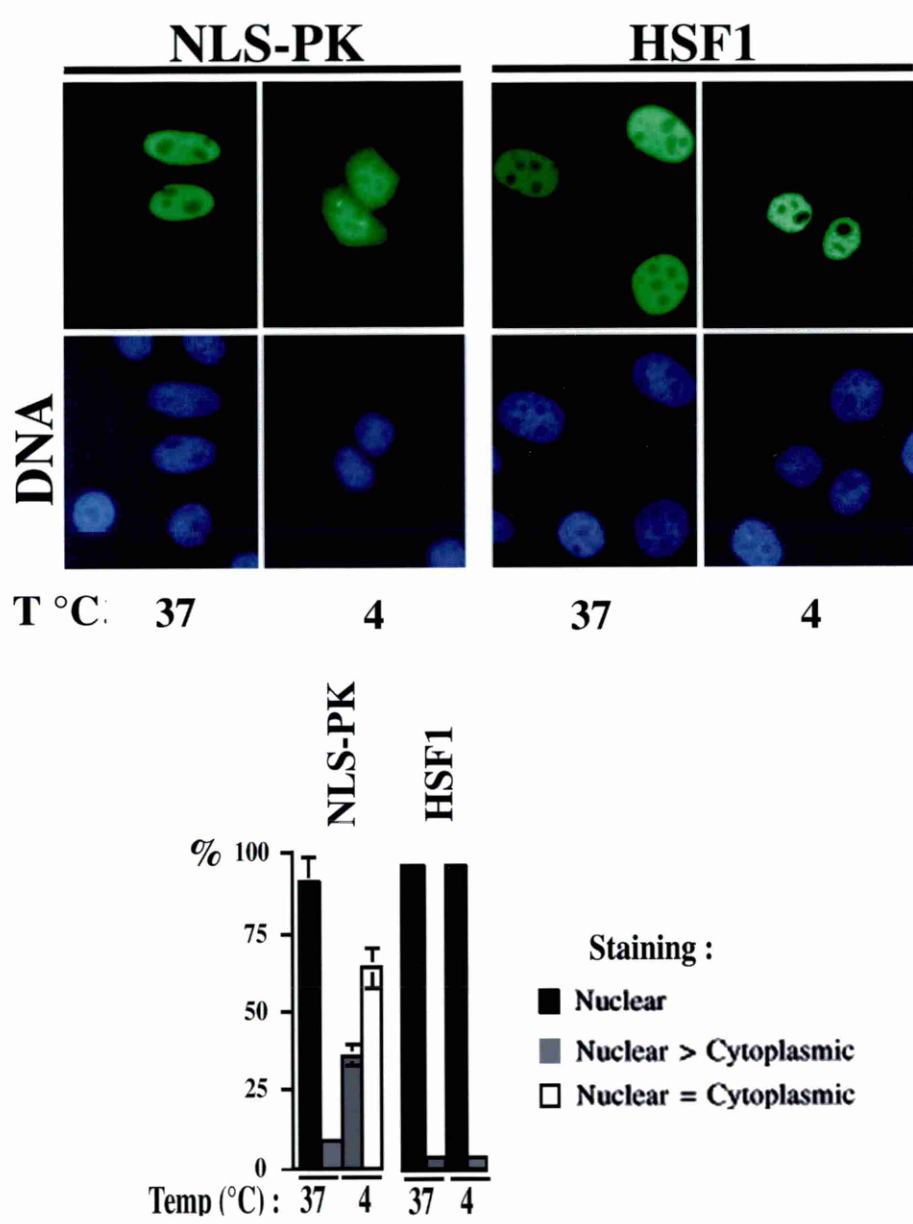
predicted molecular mass of mouse HSF1 is 55 kD, while under denaturing conditions (SDS-PAGE), HSF1 migrates as a polypeptide of 68 kD. This difference is partially due to post-translational modification i.e. phosphorylation (Sarge *et al.*, 1993). Interestingly, even when the molecular mass of both inactive and active HSF1 (either from a crude extract or as recombinant proteins) was assayed under native conditions (gel filtration chromatography), both inactive and active HSF1 were found to behave as larger species. As mentioned above, it is believed that part of this difference stems from the asymmetric, elongated shape of both forms of HSF1. Since the results described so far suggest that the inactive, monomeric form of HSF1 undergoes continuous nucleocytoplasmic shuttling, it is unlikely that a molecule with such properties would freely pass the NPC, since its shape probably increases the functional size *in vivo*. In addition, the predicted molecular mass of GFP-HSF1 is 80 kD (this fusion protein under denaturing conditions has an apparent molecular mass above 100 kD), making it unlikely that it freely diffuses through the NPC. Concerning the export of proteins from the nucleus, the actual mode of energy consumption during signal-mediated export is still a matter of some debate in the transport field. However, it is established that the translocation of cargo/receptor complex through the NPC is for signal-mediated transport a temperature-sensitive process that is attenuated or inhibited at low temperature (Nakielnny and Dreyfuss, 1999). Thus, by simply lowering the temperature to 4°C and looking at the

subcellular distribution of a nuclear shuttling protein, one can gain insight into the possible mode of nuclear export ("cooling assay", Figure 23). To test HSF1 behaviour in the cooling assay, clone 36 cells and HeLa cells transiently expressing myc-tagged chicken piruvate kinase fused to SV40 large T antigen NLS (myc-NLS-PK) (Ossareh-Nazari *et al.*, 1997), were after a brief equilibration on melting ice, incubated at 4°C for 3 hours. The incubation was done in the presence of cycloheximide to block possible residual *de novo* protein synthesis. After that, cells were fixed and processed for immunostaining. Representative staining showing subcellular distribution of myc-NLS-PK and HSF1 are shown in Figure 24. The results show that, although it is a nuclear protein at 37°C, myc-NLS-PK redistributes throughout the cell after 3 hours at 4°C, as described previously by Michael and colleagues (1995). This is most probably due to passive efflux of NLS-PK, which continued at low temperature, a condition that blocks re-import. In contrast, as demonstrated in Figure 24, HSF1 remained entirely nuclear at 4°C. Nuclear persistence of HSF1 was not altered even after prolonged incubation at 4°C (up to 6 hours), indicating that HSF1 most probably does not exit the nucleus by passive diffusion. This result argues in favour of active, signal mediated export of HSF1. Whether this occurs through direct interaction with an export receptor or NPC components or through interaction with a NES-containing adapter ("piggy-back" mechanism) remains to be determined. Therefore, taking all this in consideration, a



**Figure 23.** The cooling assay. Schematic presentation of the main steps in signal-mediated protein export and their temperature dependence (see text for details). The drawing is based upon Nakielny and Dreyfuss, 1999.

Figure 24



**Figure 24. Nuclear exit of HSF1, unlike NLS-Pyruvate kinase (NLS-PK) is blocked at low temperature.**

HeLa cells transiently overexpressing myc-tagged NLS-PK and clone 36 cells were maintained at 37°C or incubated at 4°C for 3 hours. The immunostaining was done with anti-myc and anti-HSF1 antibody, respectively. The corresponding histograms represent result from three separate experiments obtained by scoring >300 cells and classifying them using the criteria described above (see Figure 10).

systematic approach is needed to investigate the nuclear export activity in HSF1.

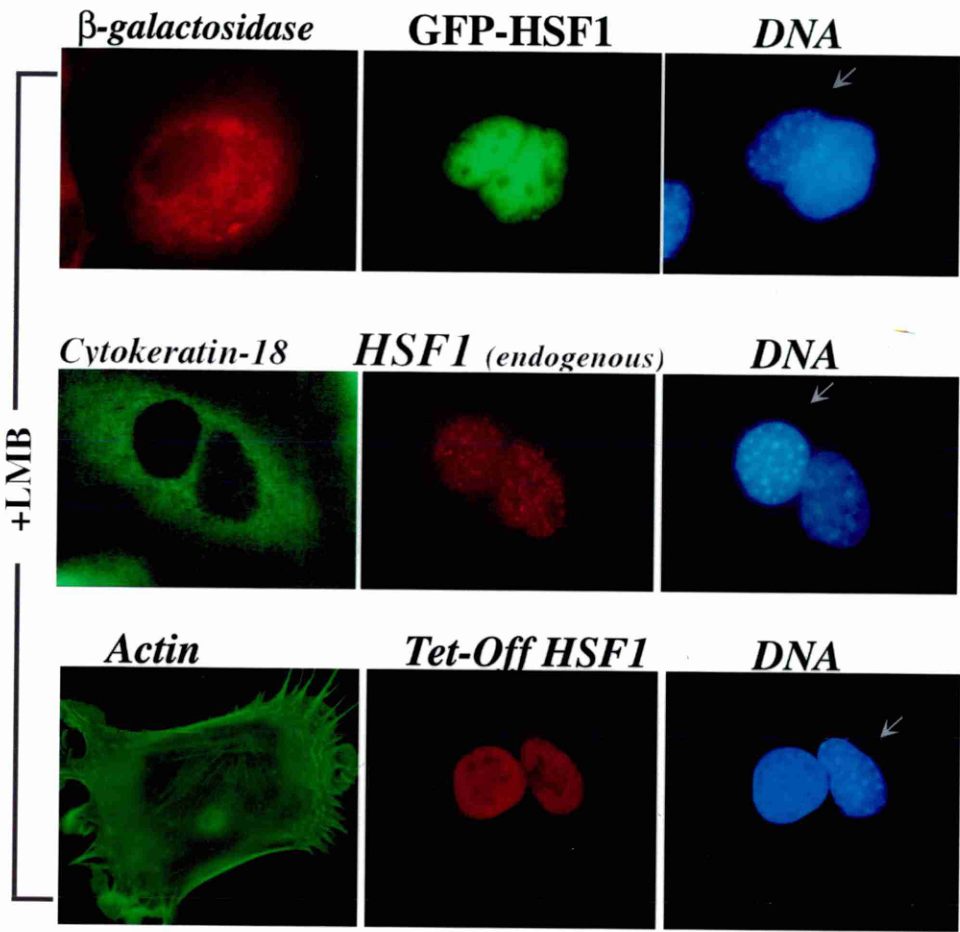
The first signal shown to mediate protein export from the nucleus was discovered in HIV Rev protein (Fischer *et al.*, 1995) and in the small cAMP-dependent protein kinase inhibitor (PKI) (Wen *et al.*, 1995). This signal is characterized by a short stretch of hydrophobic amino acids rich in leucines. It has been shown that a fungal metabolite, Leptomycin B (LMB) blocks leucine-rich NES mediated protein export in mammalian cells (Wolff *et al.*, 1997). This in turn helped to identify Crm1 (Exportin 1) as an export receptor that recognizes directly leucine-rich NES (Fornerod *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Fukuda *et al.*, 1997) and which is directly bound and modified by LMB (Kudo *et al.*, 1998). Therefore, it was important to investigate the possible effect of LMB on HSF1 to find out whether Exportin 1 is involved. Moreover, sequence analysis showed that HSF1 does not contain a consensus leucine-rich NES. However, it revealed the existence of several stretches of hydrophobic residues, within the trimerization domain of HSF1, that are rich in leucine and resemble a leucine-rich NES. Would these act as NES one would expect LMB to block nucleocytoplasmic shuttling of HSF1 in the heterokaryon assays. To test this, cocultures of HeLa cells expressing GFP-HSF1 and G2b2 mouse fibroblasts, HeLa or clone 36 cells and HSF1<sup>-/-</sup> fibroblasts were pretreated for 2 hours with different concentrations of LMB (previously shown to efficiently block leucine-rich NES mediated protein export). Cells were then fused and LMB



treatment continued for an additional 4 hours. As control, the cells were treated with the appropriate dilution of solvent (DMSO or absolute ethanol). The heterokaryons were fixed and inspected. Three representative heterokaryons after 6 h treatment with 100nM LMB for each type of HSF1 analyzed (transiently expressed GFP-HSF1, endogenous HSF1 in HeLa cells and "Tet-Off"HSF1) are shown in the Figure 25. This experiment shows that nucleocytoplasmic shuttling of HSF1 is refractory to LMB. The result was similar with 40, 100, 200 or 400nM LMB.

At this point, it was important to determine whether LMB functions in the cell under the conditions used in this study. To test this, the subcellular distribution of an endogenous protein, MAP kinase kinase (MEK1) was examined in both HeLa cells and cell hybrids before and after LMB treatment. MEK1 is also a shuttling protein, which contains a leucine-rich NES and is predominantly localized in the cytoplasm of mammalian (Fukuda *et al.*, 1996). Indeed, when the non-treated HeLa cells or cell hybrids were stained with anti-MEK1 antibody, MEK1 was found predominantly in the cytoplasm (Figure 26A). However, in both cases a treatment with 4 nM LMB for 2 hours caused nuclear accumulation of MEK1 (Figure 26A), demonstrating that, under the conditions used, LMB is functioning in the cell. Since LMB did not block internuclear transfer of HSF1 in heterokaryons, this result rules out an involvement of Exportin1. In addition, transient overexpression of Exportin 1 in HeLa cells did not induce a change in steady state localization of endogenous HSF1 24

Figure 25



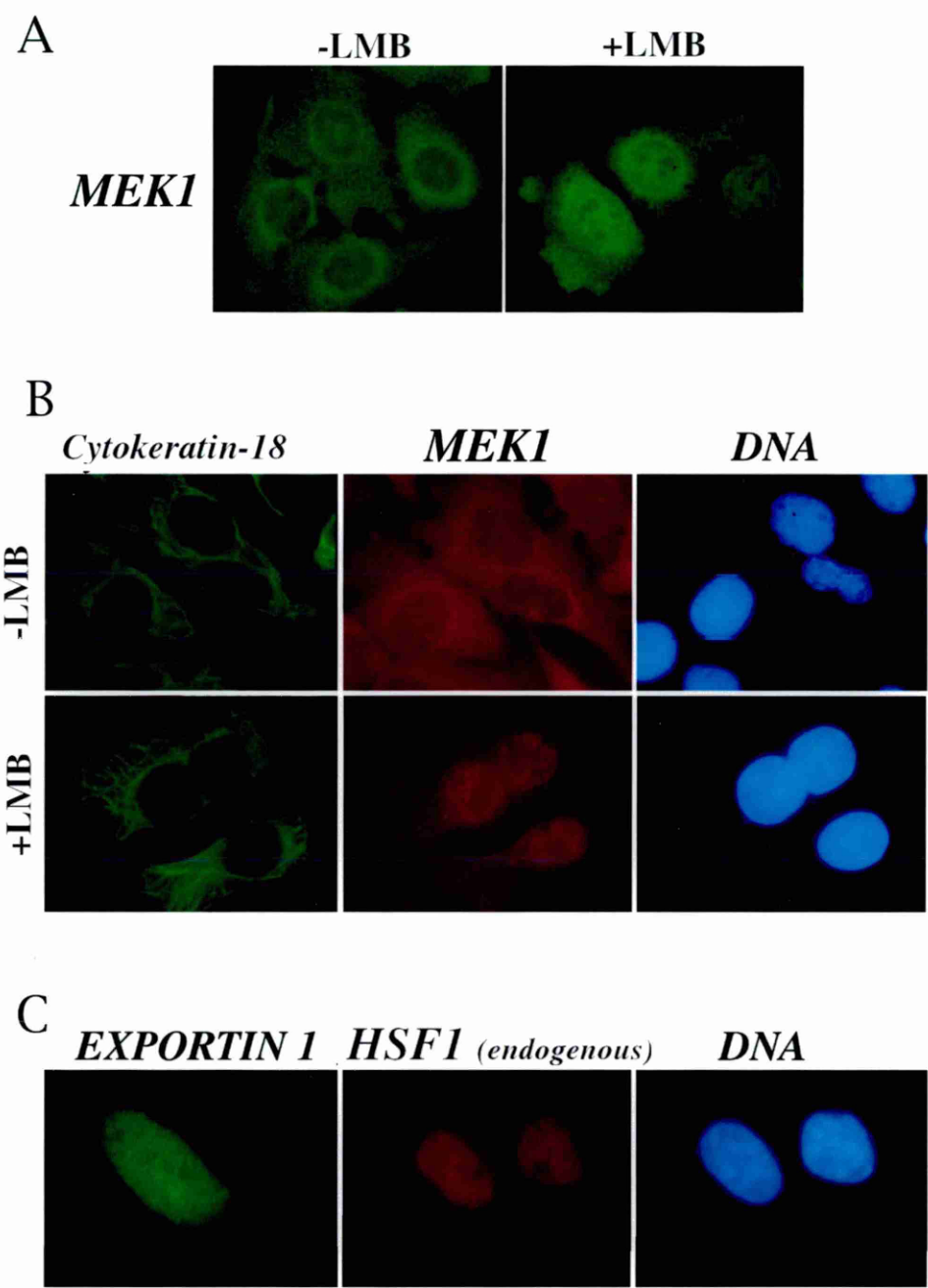
**Figure 25. Nuclear export of HSF1 is refractory to LeptomycinB (LMB).**

Nucleocytoplasmic shuttling of GFP-HSF1, endogenous HSF1 or HSF1 stably expressed in "Tet-Off" system is refractory to LMB. The cells were incubated 2 hours pre- and 4 hours post-fusion with 100nM LMB. Identical results were obtained with 40, 200 and 400 nM LMB. The arrows point to the mouse nuclei.

hours post-transfection (Figure 26B). Overexpression of Exportin 1 has been previously shown to reverse the nucleocytoplasmic ratio of some of its substrates such as NF-AT4 (Zhu and McKeon, 1999) or liver-specific transcription factor HNF-4 (Soutoglou *et al.*, 2000). HSF1 export from the nucleus may occur through a different export pathway. Therefore, it is crucial to identify sequence requirements for nuclear export activity of HSF1, which in turn would help to elucidate the export pathway. This approach would eventually lead to a better understanding of the functional relevance of continuous nucleocytoplasmic trafficking of HSF1 in mammalian cells (see Discussion).

Sequence analysis also ruled out that HSF1 contains homologies to either of the two other NES motifs described, i.e. M9 and KNS. In addition, these two signals seem to be specific for some classes of RNA-binding proteins. Two pieces of data suggested that nuclear export activity of HSF1 might reside in the trimerization domain. The first came from analysis of the subcellular distribution of carboxy-terminal deletion mutants of HSF1. When the distribution of a carboxy-terminal deletion set was analyzed in transiently transfected HeLa cells, an interesting behaviour was observed: when residues between positions 228 and 198 were deleted, HSF1 was distributed in both the nucleus and the cytoplasm. However, in almost 30% of the transfected cells, the protein was predominantly cytoplasmic (Figure 10A, deletion mutant 1-198). When deletions progressed towards the amino-terminus the percentage of the cells showing cytoplasmic

Figure 26



**Figure 26. Leptomycin B blocks nuclear export of endogenous MAP kinase kinase (MEK1) in HeLa cells and in transient HeLa-HeLa homokaryons.**

(A) HeLa cells were either, treated 2 hours with 4 nM LMB and then analyzed by immunofluorescence with polyclonal anti-MEK1 antibody (upper panel) or treated 2 hours pre- and 2 hours post-fusion with 20 nM LMB. In both cases, controls (-LMB) included incubation with appropriate dilution of LMB solvent.

(B) **Overexpression of myc-tagged Exportin 1 does not induce change in steady state localization of endogenous HSF1 in HeLa cells.** The immunostaining was performed 24 hours post-transfection.

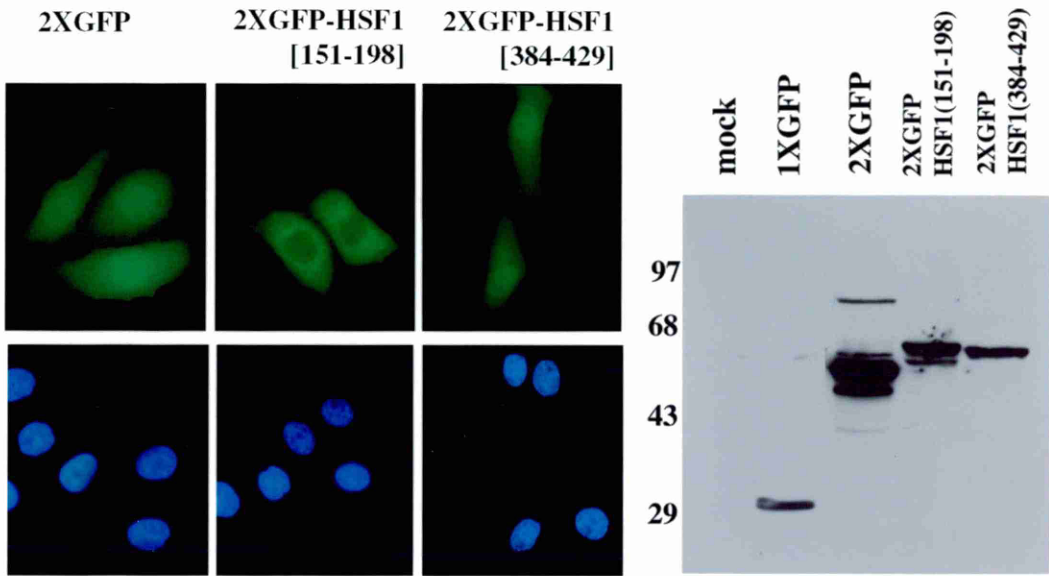
localization decreased and further deletion up to residue 120 resulted in an apparently equal distribution of HSF1 between the two compartments (Figure 10A, deletion mutants 1-167, 1-141 and 1-120). This was reproducibly seen in several independent experiments and pointed out a clear shift towards more cytoplasmic residency (compare histograms corresponding to deletion mutants 1-198 and 1-140 in Figure 10A). Although the distribution of these mutant species could result from passive diffusion between the nucleus and the cytoplasm due to their small sizes (~20kD), this behaviour was also seen with the corresponding GFP fusions (data not shown). Therefore, the possibility that a sequence required for export activity of HSF1 resides in this region was investigated. When the region of HSF1 between these deletion points (151-198) was fused to a reporter protein, dimerized GFP (GFP2), which is diffusely distributed throughout HeLa cell, GFP2-HSF1 (151-198) was predominantly detected in the cytoplasm (Figure 27A). A region of comparable length and containing a similar structural motif, the carboxy-terminal leucine zipper (residues 384 to 429) did not have this effect on GFP2 (Figure 27A). The predominant cytoplasmic distribution of GFP2-HSF1 (151-198) was not due to the trimerization function of residues 151-198, because this was not altered if the trimerization ability of HSF1 was abrogated by deleting residues 160-172 (data not shown). Analysis of protein extracts from transiently transfected HeLa cells with anti-GFP antibody demonstrated that all polypeptide species were intact and of predicted mass (Figure 27A). These data suggest

that residues 151 to 198 from the trimerization domain induced an apparent change in the steady state subcellular distribution of GFP2. Whether this effect is really due to the export activity was examined using the heterokaryon assay. In this experiment a non-shuttling protein myc-tagged nucleoplasmin core protein driven to the nucleus by SV40 large T antigen NLS (myc-NPc-TNLS), was used. This reporter protein was shown not to shuttle in the heterokaryon assay; however, it can be converted to a shuttling protein by fusing an NES (Michael *et al.*, 1995). Therefore, it was reasonable to test a putative export activity of candidate regions of HSF1 by fusing them to NPc-TNLS and analyzing the fusion constructs in the heterokaryon assay. For this purpose, a fusion protein, GFP-NPc-TNLS was made and, as expected, shown not to shuttle in the heterokaryon assay 4 hours post-fusion (data not shown). Surprisingly, when the candidate region of HSF1 was fused to this reporter, the fusion protein GFP-NPc-TNLS-HSF1(151-198) showed diffuse distribution throughout the cell in >80% of transiently transfected HeLa cells 24 hours post-transfection (Figure 27B), even when its trimerization ability was abrogated by deletion of residues 160 to 172 in construct GFP-NPc-TNLS-HSF1(151-198 $\Delta$ 160-172), (data not shown). Consistently, the addition of the 47 residues from the carboxy-terminal leucine zipper to the basic reporter construct GFP-NPc-TNLS did not alter its nuclear residency, since the construct GFP-NPc-TNLS-HSF (384-429) remained nuclear in most of the transfected cells (Figure 27B). Unfortunately, since the addition of residues 151 to 198

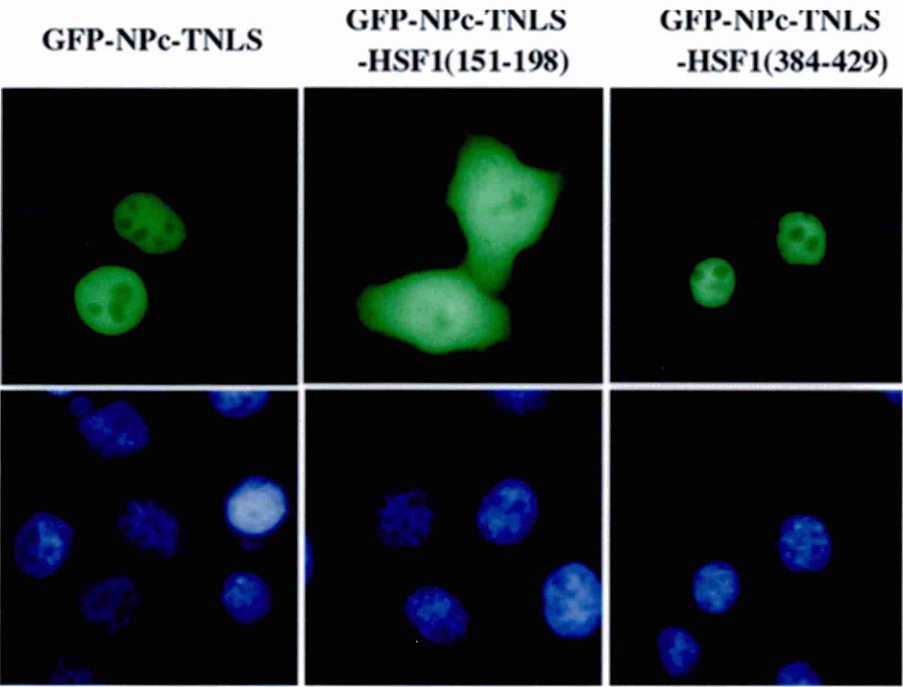


Figure 27

A



B



**Figure 27. A region from the trimerization domain of HSF1 induces an apparent shift in nucleocytoplasmic distribution of reporter proteins.**

(A) Subcellular distribution of dimerized GFP, (GFP2) or GFP2-HSF1 fusion proteins, GFP2-HSF1(151-198) and GFP2-HSF1(384-429), in transiently transfected HeLa cells. On the right is shown immunoblot on whole cell extracts with anti-GFP antibody.

(B) Subcellular distribution of GFP-tagged nucleoplasmin core fusion proteins, GFP-NPc-HSF1(151-198) and GFP-NPc-HSF1(384-429) in transiently transfected HeLa cells.

resulted in the diffuse distribution of the reporter protein in the cells, this reporter protein could not be used in the heterokaryon assay since the heterokaryon assay is based on the detection of internuclear protein transfer. Therefore, ambiguities, which these proteins may give, made them not reliable reporters. Interestingly, very similar effect of the leucine-rich NES from the shuttling protein 4E-T on the subcellular distribution of NPc-TNLS was described recently (Dostie *et al.*, 2000). In this case as well, addition of the leucine-rich NES to the NPc-TNLS resulted in NPc-TNLS-NES being distributed between the nucleus and the cytoplasm. However, it remains unclear whether the altered subcellular distribution of reporter proteins fused to the trimerization domain of HSF1 is a result of nuclear export activity or of a spurious interaction with some cytoplasmic component that retains these reporters out of the nucleus. Thus, direct evidence must be obtained using other transport assays i.e. mammalian cell or *Xenopus* oocyte microinjection. Currently, work is ongoing to construct other reliable reporter molecules to be tested in the heterokaryon assay.

The second piece of data that suggested that nuclear export activity of HSF1 might reside in the trimerization domain was obtained when the internuclear transfer of GFP-HSF1: $\Delta$ 160-172 was investigated. This deletion results in a constitutively monomeric HSF1, as demonstrated in Figure 9C. Surprisingly, internuclear transfer of this mutant was quite delayed with respect to the wt HSF1 because 4 hours post-fusion, no internuclear equilibration can be detected (Figure

20), whereas wt HSF1 equilibrates between the nuclei of nearly 50% of heterokaryons (Figure 15A). The reason for this is unclear, but it is tempting to speculate that this deletion might weaken but not abolish the nuclear export activity of HSF1.

Taken together, these results suggest that a region responsible for nuclear export activity of HSF1 might reside within the trimerization domain. However, at this point only additional constructs with more mutations in the trimerization domain of HSF1 can help to elaborate the basis for a possible involvement of the trimerization domain of HSF1 in nuclear export.

## DISCUSSION

### Nucleocytoplasmic shuttling of HSF1

This work shows that mammalian HSF1, a transcription factor that regulates stress-induced expression of heat shock protein coding genes, is not confined to the nucleus. By indirect immunofluorescence analysis of mammalian tissue culture cells, HSF1 was shown to be predominantly nuclear before and after heat stress (Rabindran *et al.*, 1993; Cotto *et al.*, 1997; Jolly *et al.*, 1998; Mercier *et al.*, 1998; Figure 6). However, the data presented here reveal a dynamic behaviour of HSF1, demonstrating its constant nucleocytoplasmic shuttling (Figure 13). The continuous flux of HSF1 between the two compartments occurs at physiological temperature (when HSF1 is kept as an inactive monomer) with a minimum export rate of approximately 1 molecule/second. Shuttling is discontinued during mild heat stress (when HSF1 is converted to its active, trimeric form), but resumes during recovery (when HSF1 trimers dissociate back to monomers). Moreover, shuttling is also impaired at 37°C for deregulated (oligomeric) HSF1 mutants with mutations that derepress oligomerization. Taken together, these data are consistent with shuttling of inactive, monomeric

HSF1 as presented in a possible model for HSF1 transport cycle shown in Figure 21.

Two lines of evidence presented in this work suggested that both nuclear import and nuclear export of HSF1 occur by an active (signal-mediated) process. First, a bipartite nuclear localization signal was identified and second, although the sequence requirements for nuclear export activity have not been delineated, nuclear confinement in the cells incubated at 4°C is consistent with export being an active process. Interestingly, Leptomycin B did not inhibit nucleocytoplasmic shuttling suggesting that HSF1 nuclear export occurs through a distinct pathway. The finding that HSF1 constitutively shuttles between the nucleus and the cytoplasm may have implications for HSF1 regulation and it is also of interest for understanding how proteins are imported into and exported from the nucleus.

Over the past several years, a number of reports described the dynamic behavior of many nuclear proteins which, like HSF1, continuously move back and forth across the nuclear envelope. Among them, two transcription factors, progesterone receptor (Guiochon-Mantel *et al.*, 1991) and p53 (Roth *et al.*, 1998; Stommel *et al.*, 1998) were shown to shuttle in heterokaryon assay. In addition, glucocorticoid receptor, although at steady state cytoplasmic, has also been shown to shuttle in the heterokaryon assay

(Madan *et al.*, 1993). With respect to the apparent rate of internuclear transfer, these proteins behave differently. Progesterone receptor apparently equilibrates between the nuclei in heterokaryons within 12 hours post-fusion, whereas p53, HSF1 and glucocorticoid receptor exhibited faster shuttling kinetics: p53 apparently equilibrates between the nuclei in heterokaryons within 3 hours, whereas HSF1 and glucocorticoid receptor apparently equilibrate in the nuclei of heterokaryons 4 hours post-fusion. Of the two other nuclear transcription factors tested here for shuttling in the heterokaryon assay, Crem $\tau$  displayed kinetics of internuclear equilibration very similar to that of HSF1 or p53 (Figure 17), while in contrast, c/EBP- $\alpha$  showed little, if any, internuclear transfer within the time frame used (Figure 14). Based on this analysis, it seems that nucleocytoplasmic shuttling of nuclear transcription factors may be a more general phenomenon and that differences in the kinetics of shuttling could reflect the differences in their regulatory pathways. This is certainly an issue of great interest and perspective as more and more transcription factors are being classified as shuttling proteins.

The phenomenon of nucleocytoplasmic shuttling is in many cases mediated by specific signals in the proteins-nuclear localization signal (NLS) for nuclear import and nuclear export signal (NES) for nuclear

export (Mattaj and Englmeier, 1999). Shuttling can also be controlled by nucleocytoplasmic shuttling (NS) signals, which unlike NLS and NES can direct both nuclear import and nuclear export (Michael, 2000). Here it is shown that nuclear accumulation of HSF1 is driven by a bipartite nuclear localization signal, comprising 25 amino acids (residues 203-228) and consisting of two modules (Figures 10 and 11). The first, located in the trimerization domain, contains three clustered basic residues (lysine, arginine and lysine at position 206-208) and plays a major role in nuclear targeting of HSF1. When these residues were changed to alanines, nuclear accumulation of HSF1 was abolished (Figure 11). The exact boundaries of the second module, 15 residues apart, are not apparent, since there are no clustered basic amino acids. However, lysine at position 224 and arginine at position 227 apparently contribute to nuclear accumulation, although not to the same extent as three clustered basic residues. When these residues were changed to alanines a portion of the protein remained cytoplasmic, although the bulk of HSF1:K224A/R227A was nuclear (N>C) (Figure 11). This suggests that the two modules may not function interdependently.

The NLS of HSF1 differs from the classical bipartite NLS, since two modules have an unusual spacing of 15 instead of 10 residues and by the fact



that the second, carboxy-terminal module does not contain clustered basic residues which are usually found in a bipartite NLS. Instead, the second module contains two basic residues, lysine and arginine, separated by two amino acid insertions. In addition, in the classical bipartite NLS, the two modules are usually interdependent, which does not seem to be the case for HSF1 NLS. However, the mutational analysis of the NLS presented here is at the moment partial and more experiments are ongoing to complete its characterization. These experiments are going in two directions. First, mutagenesis experiments will help to assign the contribution of each of the basic residues from both modules (as well as a possible role of other amino acids in the spacer region) in nuclear targeting. Second, the NLS will be tested for *in vitro* interaction (using a GST-pulldown assay) with importin  $\alpha$  to address whether HSF1 utilizes "classical" import pathway mediated by an importin  $\alpha/\beta$  heterodimer. If the NLS of HSF1 does not interact with importin  $\alpha$ , it is possible that importin  $\beta$  is responsible for the nuclear import of HSF1. Alternatively, import of HSF1 is mediated by an unknown import receptor. In that case an alternative strategy can be considered for identifying such a receptor by using, for example, a genetic screen (yeast two hybrid assay) or biochemical assay (affinity chromatography). Recently, it was reported that a

region in human HSF1 (comprising residues 203-224) overlapping with the NLS described here, is required for JNK protein kinase targeting as well as for nuclear targeting of human HSF1 (Dai *et al.*, 2000). When this region (highly conserved between human and mouse HSF1) was deleted, human HSF1 was relocalized to the cytoplasm of transfected cells providing evidence that this region contains the NLS.

Considering the nuclear exit of HSF1, the data presented in this work point to an active, signal-mediated export mechanism. The results of the cooling assay clearly showed that HSF1 is trapped in the nucleus when the cells are incubated at 4°C (Figure 24). This indicates (since the signal-mediated nuclear export is temperature-dependent) that nuclear exit of HSF1 is most likely signal-mediated. Moreover, since shuttling of HSF1 is refractory to Leptomycin B (Figure 25), this suggests that the export pathway HSF1 utilizes is distinct from the one mediated by the Exportin1. Apart from the leucine-rich NES, the only other NES motifs described so far were identified in certain RNA-binding proteins where they also function as NLS (Michael *et al.*, 1995, 1997). However, sequence comparison showed no similarities between these signals (M9 and KNS) and the HSF1 primary sequence. Therefore, it is possible that the nuclear export activity of HSF1 is due to the presence of a novel NES within the protein. Alternatively, HSF1

could be "piggy back" exported to the cytoplasm through its association with an NES containing adapter. This mode of export from the nucleus has already been reported for some proteins (Arenzana-Seisdedos *et al.*, 1997). Several experiments presented here suggest that the nuclear export activity of HSF1 may reside within the trimerization domain. First, the analysis of the subcellular distribution of myc-tagged carboxy-terminal deletion mutants of HSF1 (Figure 10), indicates that the residues at position 167-198 may contain nuclear export activity because addition of this region shifts nucleocytoplasmic distribution of the respective deletion mutants, making mycHSF1(1-198) more cytoplasmic than mycHSF1(1-167). Second, when this region of HSF1 (residues 151-198) was fused to either dimerized GFP, or nucleoplasmin core protein (NPc) containing SV40-TNLS, it also caused cytoplasmic redistribution of these proteins (Figure 27). Interestingly, a similar effect was obtained by fusing the leucine-rich NES of the shuttling protein 4E-T to the NPc-TNLS (Dostie *et al.*, 2000). In contrast, control region of HSF1 containing carboxy-terminal leucine zipper, (residues 384-429), when fused to dimerized GFP or NPC-TNLS did not alter their nuclear residency (Figure 27). However, these data are showing subcellular distribution in the transfected cells at steady state and the possibility that this localization may arise from a spurious

interaction with some cytoplasmic component can not be formally excluded. Thus, to obtain direct evidence for nuclear export activity, this sequence should promote the export of heterologous proteins (that would otherwise be restricted to the nucleus) using some of the *in vivo* protein nuclear export assays (heterokaryons, *Xenopus* oocyte nuclear injection or somatic cell nuclear injection). These experiments are currently ongoing and they will be crucial in the identification of the sequence requirements for the nuclear export activity of HSF1. The next step will be the identification of the export receptor responsible for HSF1 export from the nucleus.

## **A possible role of nucleocytoplasmic shuttling in the regulation of HSF1**

It is becoming evident that the activity of many transcription factors can be regulated at multiple levels. Numerous studies have demonstrated that one of the important levels of regulation could be the regulation of subcellular localization. In certain cases, as described in the introduction, transcription factors are kept in a latent inactive form in the cytoplasm and relocate to the nucleus upon a specific signal. In the nucleus they activate transcription of a specific set of target genes. The mechanism by which these proteins are kept in the cytoplasm is mostly through the masking of their NLS, which is thus not available to the import machinery. In contrast, other transcription factors shuttle constantly in and out of the nucleus and are at steady state localized either in the nucleus, in the cytoplasm or in both compartments. In this case, the steady-state subcellular distribution is mostly determined by the differences in the rates of import and export. Here, evidence is presented that HSF1 shuttles continuously in and out of the nucleus at physiological temperatures. However, the net result of this flow results in a very asymmetric distribution of the protein at steady state. Nuclear accumulation is probably due to a higher import rate, as indicated in

a possible model of HSF1 shuttling cycle shown in Figure 21.

Importantly, the cycling of HSF1 is regulated by heat shock. Under conditions of mild heat stress, when HSF1 is converted to a trimer, export is reversibly discontinued (Figures 15B and 16) and HSF1 is confined to the nucleus. This block of nuclear export under conditions of heat stress is specific for HSF1. Under the experimental conditions used, the internuclear transfer of another shuttling protein, CREM $\tau$  was not impaired (Figure 17). Furthermore, a similar block of shuttling was detected for HSF1 mutants (H179R and M387K/L391A/L394A) bearing amino acid substitutions that relieve the suppression of oligomerization under normal growth conditions. Both mutants were oligomeric at 37°C and showed impaired internuclear transfer in the heterokaryon assay (Figure 18), thus recapitulating the effect of heat stress on shuttling of wild type HSF1. It is intriguing that two mutations, which are located in distinct protein domains (H179R in the trimerization domain and M387K/L391A/L394A in the carboxy-terminal leucine zipper 4) gave a very similar phenotype in the shuttling assay. These two regions of HSF1 have been proposed to maintain HSF1 monomeric through an intramolecular coiled-coil interaction (Rabindran *et al.*, 1993). It is believed that during heat stress, this interaction is perturbed

and protein oligomerizes through the intermolecular coiled-coil interaction between the trimerization domains. Therefore, the most obvious explanation for the observed block of shuttling of oligomeric HSF1 would be masking of NES activity in the trimerization domain. This would prevent the interaction of this region of HSF1 with the export machinery and trap the protein in the nucleus. However, this mechanism remains to be demonstrated and is not the only possible way to regulate nuclear export of HSF1. It is not clear whether this masking is due solely to the interaction between trimerization domains or also with other nuclear components. So far, as mentioned in the introduction, it has been reported that HSF1 interacts *in vivo* with hsp70, hsp90 and HSBP1. Hsp70 was found to interact with both inactive and active HSF1, while hsp90 was captured in a complex with inactive HSF1. Although in the case of hsp70 the data obtained by two different groups were not consistent, interactions with both hsp70 and hsp90 were proposed to repress HSF1 activation under physiological conditions. Under these conditions, HSF1 would exist in a dynamic complex with chaperones. During heat stress, non-native proteins accumulate and compete with HSF1 for binding to hsp70 or hsp90. As a result, the HSF1-chaperone complex will dissociate, the concentration of free HSF1 would increase and HSF1 oligomerization would

be favoured. During recovery from the stress conditions, HSF1 would dissociate back to the monomeric form. Dissociation would be favoured under the conditions of decreased concentration of nonnative proteins (which are either refolded or targeted to degradation by the action of the chaperones), and the HSF1-chaperone complex(es) would be again formed. Although there is no conclusive evidence for this, the data obtained so far strongly suggest involvement of chaperones in regulation of HSF1. Besides their proposed role in repression of HSF1, the possibilities that chaperones are involved in nucleocytoplasmic shuttling can not be excluded. However, to fully address these questions, more characterization of HSF1 and hsp70/hsp90 is awaited.

Hsp70 is mainly a cytoplasmic at 37°C and relocalizes to the nucleus upon heat stress (Welch, 1993). Hsp90 is distributed in both the nucleus and the cytoplasm (Gasc *et al.*, 1990; Yang *et al.*, 1997). Unlike hsp70, its distribution does not change after heat shock. Both proteins are, unlike HSF1, very abundant and a substantial fraction of them exists in the cell either in the form of multichaperone complexes or in association with other proteins. In the light of shuttling of HSF1, it is worth noting that hsp70 itself is a shuttling protein (Mandell and Feldherr, 1990). In addition, it has been demonstrated



that hsp70 is required for nuclear import of some proteins, using *in vitro* assay with permeabilized cells (Shi and Thomas, 1992). In addition, there is genetic evidence in yeast that hsp70 acts during both targeting and translocation phases of nuclear import (Shulga *et al.*, 1996). Probably, hsp70 assists formation of some import receptor/cargo complexes. On the other hand, there is no evidence that hsp90 can shuttle between the nucleus and the cytoplasm, but it is interesting that the treatment of tissue culture cells with metal oxyanions, such as molybdate, stimulate *in vitro* nuclear export of hsp90 (Yang *et al.*, 1997). The nuclear export of hsp70, glucocorticoid receptor and hnRNPA1, but not of hsp56 and hnRNPC, was also stimulated. Since hsp70 and hnRNPA1 are, unlike hnRNPC, shuttling proteins *in vivo*, it is possible that metal oxyanions affect a component of the nuclear export pathway for common shuttling proteins.

HSBP1 was also proposed to negatively regulate HSF1 (Santyal *et al.*, 1998). HSBP1 is a nuclear protein that interacts *in vivo* with the active trimeric HSF1 and also associates with hsp70 during the attenuation of the heat shock response when HSF1 trimers disassembled back to monomers. These results did not exclude the possibility that HSBP1 interacts with inactive, monomeric HSF1 and plays an additional role

in HSF1 regulation, for example in nucleocytoplasmic shuttling.

The results presented here point to the masking of nuclear export activity as the most probable reason for the nuclear confinement of oligomeric HSF1. It is interesting that a very similar mode of nucleocytoplasmic shuttling regulation has been proposed for p53, in which monomeric p53 shuttles in and out of the nucleus under normal conditions and its subcellular distribution varies during the cell cycle. Under stress conditions, p53 tetramerizes and tetramerization masks the leucine-rich NES (which overlaps precisely with the tetramerization domain), thus preventing its export (Stommel *et al.*, 1998). In this way, tetrameric p53, which is transcriptionally active, remains nuclear. This may have some functional significance since in many of p53-related tumors p53 is relocalized to the cytoplasm (Sun *et al.*, 1992). Although direct evidence for this type of regulation in the case of HSF1 is missing, HSF1 was also found to be in part misslocalized in some tumors (Hoang *et al.*, 2000). Whether this has to do with the change in the rates of import and/or export of HSF1 remains to be determined. Possibly, the rates of nuclear import and export of HSF1 are subjected to regulation *in vivo*. It has been demonstrated that post-translation modifications (phosphorylation or acetylation) can influence the rate of transfer across

the nuclear envelope of many proteins, such as, for example nucleoplasmin, NF-AT, HNF4 or cyclin B1 (Vancurova *et al.*, 1995; Zhu *et al.*, 1998; Li *et al.*, 1997). This is probably achieved by regulating the association of a protein with its import/export receptor (Kaffman *et al.*, 1998).

As mentioned above, HSF1 is a phosphoprotein and becomes hyperphosphorylated during heat stress. Basal phosphorylation of HSF1 (at residues 303 and 307) was so far demonstrated to play a role in suppressing transcriptional activity under physiological conditions (Chu *et al.*, 1996; Kline and Morimoto, 1997). Moreover, the exact role of stress-induced hyperphosphorylation, as well as the identity of the phosphorylated residues, remains unsolved. It is possible that phosphorylation is important in HSF1 trafficking.

Why does HSF1, accumulated at steady state in the nucleus, where it exerts its biological function, need to be constantly exported to the cytoplasm? There are two possible answers to this question. First, bidirectional movement of HSF1 through the NPC may play a role in HSF1 regulation. Second, HSF1 shuttling is a futile cycle. The logic and especially the growing number of reports in the nucleocytoplasmic transport field, however, argue in favor of the first possibility. Several scenarios can be envisaged. First, nuclear export of HSF1 at physiological conditions may be

involved in monomer maintenance. This could be achieved through the interactions of HSF1 with some cytoplasmic components that contribute to the suppression of trimerization. The candidates for the cytoplasmic interactors could be either enzymes that modify HSF1, or chaperones. For example, either compartment-specific post-translational modification, or transient association with chaperones could be important. If these interactions are perturbed during stress conditions, this would destabilize the monomer and oligomerization will be favoured. Furthermore, HSF1 monomers may be "sensing" the cytoplasmic environment, and detecting sustained forms of stress. Unlike heat stress, which is a severe and acute experimental stress, it is possible that *in vivo* HSF1 is capable of responding to more subtle changes in cell physiology. Thus, by monitoring the cytoplasm, (perhaps through transient interactions with proteins/enzymes or with a small ligand or a metabolite), HSF1 can "sense" the changes induced by sustained stress signals. The interactions with these components would somehow "modify" HSF1 and trigger or set thresholds for its activation. Once "modified" in the cytoplasm, HSF1 would be reimported into the nucleus in a "pre-activated" state. These two possibilities are not mutually exclusive and together with a model presented in Figure 21 raise an important point to discuss. Since

HSF1 is exported at the minimum rate of 1 molecule/second, it is reasonable to assume that at any given moment the concentration of HSF1 monomers in the cytoplasm is very low. On the other hand, oligomerization of HSF1, as discussed above, is concentration dependent; hence the cytoplasmic pool of HSF1 is probably insufficient for the protein to oligomerize. This would imply that *in vivo* oligomerization takes place in the nucleus, although it was demonstrated that the nuclear environment is not required for HSF1 to trimerize (Orosz *et al.*, 1996). Finally, it is possible that HSF1 has an unknown role in the cytoplasm and therefore needs to be constantly exported from the nucleus. Considering its low abundance, it is unlikely that HSF1 act as a transporter of other molecules (unless the cargoes are also low abundance factors).

While the export pathways as well as the functional significance of shuttling of progesterone and glucocorticoid receptors is still obscure, nuclear export of p53 (mediated by hdm2) has been suggested to play a role in regulating the level of this protein (Roth *et al.*, 1998). It seems unlikely that the same would apply for HSF1, considering that HSF1, unlike p53, seems to be a stable protein. Rather, the export rate and stability of HSF1 (assayed under conditions when *de novo* protein synthesis is blocked by cycloheximide) implies that the whole HSF1 pool in a

HeLa cell (30,000 molecules) shuttles back and forth several times during each interphase. This is in agreement with proposed roles for HSF1 cycling, such as monomer maintenance or cytoplasmic surveillance.

Recently, dynamic changes of intracellular localization of HSFs have been found in plants. HSFA2, expressed in a stress-dependent manner is a shuttling protein (Heerklotz *et al.*, 2000) localized to the cytoplasm due to the presence of the leucine-rich NES located at the very carboxy-terminus. Both deletions of this region and LMB treatment induced nuclear accumulation of HSFA2. The sequence of the NES in HSFA2 is somewhat conserved in mouse HSF1 and is located immediately adjacent to the carboxy-terminal leucine zipper, comprising residues 410-420. However, it is unlikely that this sequence is responsible for the nuclear export of HSF1, because the shuttling of HSF1 is not sensitive to LMB. In addition, a deletion mutant, GFP-HSF1 (1-407), in which this sequence has been removed, was not impaired in shuttling (data not shown). It is therefore possible that different pathways are utilized in plant and mammalian cells for HSF nuclear export. The finding that shuttling as a phenomenon is conserved for both plant HSFA2 and mammalian HSF1, strongly argues in favour of shuttling playing a role in HSF1 functions. Whether that role will be similar in both mammalian and plant cell remains to be seen. It will be interesting to check

if shuttling is also conserved in other members of the HSF family, such as HSF2, which are subjected to other modes of regulation.

To conclude, the role for HSF1 shuttling can be properly understood only by identifying the NES and the export pathway HSF1 utilizes. After that, export-defective mutants of HSF1 can be generated and their phenotype tested (by monitoring the expression of endogenous HSF1 target genes) following restored expression in HSF1 null cells. This would ultimately help to elucidate the role of nuclear export in HSF1 regulation, which is one of the main challenges ahead in the field of heat shock response.

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